REMARKS

Claims 8, 32, and 46 have been amended. Claims 1-7, 17-31, 42-45, and 47-48 have been canceled without prejudice. New claims 49-51 have been added. Accordingly, claims 8-16, 32-41, 46, and 49-51 are now pending for the Examiner's consideration.

By this amendment claims 8, 32, and 46 are amended and claims 49-51 are added. Support for new claims 49-51 is found in the specification, *inter alia*, on pages 1-2, paragraph [0010], page 2, paragraphs [0014], [0015], [0018], and [0019], and page 4, paragraph [0043]. No new matter is added.

With respect to all claim amendments, Applicants have not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Claim Objections

Claim 1 is objected to with regard to the abbreviation "AUC" (see Office Action, p. 2). Because claim 1 has been canceled, this objection is now moot and Applicants respectfully request that it be withdrawn.

35 U.S.C. §112, 1st paragraph

a. Written Description

Claims 1-16, 17-28, 30-45, and 47 are rejected under 35 U.S.C. §112, 1st paragraph for allegedly failing to comply with the written description requirement. In particular, the Examiner asserts that the Applicants have not described with sufficient clarity the terms "solvates, active metabolites, and prodrugs thereof" (see Office Action, pp. 3-6). Of the rejected claims, claims 1-7, 17-28, 30-31, 42-45, and 47 have been canceled. In addition, independent claims 8 and 32 have been amended to remove any reference to solvates, active metabolites, and prodrugs. By the present amendments, Applicants believe this rejection has been overcome, and respectfully request that the rejection be withdrawn.

b. Enablement

Claims 17-28, 30, and 32-47 are also rejected under 35 U.S.C. §112, 1st paragraph for allegedly failing to comply with the enablement requirement. In particular, the Examiner asserts that while being enabling for treating a representative of abnormal cell growth or cancer, the specification does not enable a wide variety of abnormal cell growth or cancer (see Office Action, pp. 6). The Examiner states that due to the unpredictable nature of the pharmaceutical art, the

specification does not support the broad use of the compound of formula I for the treatment of a wide variety of abnormal cell growth. In particular, the Examiner states that there is "no common mechanism by which a large, or even most, abnormal cell growth or cancer arise" (see Office Action p. 7). Accordingly, the Examiner concludes that in the absence of a correlation between at least some of the disease states claimed as being capable of being treated by the compound of formula I, one of skill in the art would be unable to predict the possible results. For the reasons that follow, Applicants respectfully traverse this rejection.

First, Applicants wish to point the Examiner's attention to the fact that the present claims are not directed to methods of treating cancer using multiple compounds at any dosage amount. Rather, the claims are directed to the use of the ***specific compound of formula I*** (or any pharmaceutically acceptable salt thereof) using a ***specific dosage amount*** to treat cancer.

Furthermore, independent claim 32 has been amended to replace the term "abnormal cell growth" with "cancer". Applicants assert that claims to the treatment of a wide variety of cancers using the specific compound shown in formula I is supported by the specification. In particular, the compound of formula I is noted to be an inhibitor of vascular endothelial cell growth factor receptor (VEGF-R) and of platelet-derived growth factor receptor-β (PDGFR-β), both of which are targets recognized in the art to be related to angiogenesis and tumor growth (see Bergers, *J. Clin. Invest.* 111:1287-1295 (2003) – Exhibit 1). Abnormal angiogenesis is a recognized hallmark of several disease states, such as retinopathies, psoriasis, rheumatoid arthritis, age-related macular degeneration, and cancer (see Folkman, *Nature Med.* 1:27-31 (1995) – Exhibit 1). In the case of cancer in particular, angiogenesis is necessary for the progression from benign to malignant tumors, as well as the growth and metastases of malignant cells (see Folkman, *Curr. Mol. Med.* 3:643-651 (2003) – Exhibit 1). Accordingly, the use of anti-angiogenic agents to treat a variety of disease states that rely on angiogenesis is a common strategy in the pharmaceutical arts (see Senger, *Am. J. Pathol.* 149:1-7 (1996) – Exhibit 1). Thus, contrary to the Examiner's assertion, there is a common mechanism that relates to several types of cancer – namely angiogenesis. Because many cancers depend on this common mechanism of angiogenesis, it would be expected that a compound of formula I (which is known to reduce angiogenesis as an inhibitor of VEGF-R and PDGFR-β) could be used to treat a variety of cancers.

Furthermore, Applicants wish to point out data in the specification that suggests the compound of formula I can be used to treat a variety of cancers. For example, the specification includes positive results from several pre-clinical tumor xenograft mouse models, including MDA-MB-231 (human breast cancer – see page 10, paragraph [0098] and Table 3), MV522 (human colon cancer – see page 12, paragraphs [0117]-[0118]), and Lewis Lung carcinoma (lung cancer – see Example 5 on pages 11-12). In addition, the specification includes data from human clinical studies where the compound of formula I was administered in varying doses to patients with a variety of solid tumors (see Example 2 on pages 10-11) and indicates positive results for

renal cell carcinoma and adenoid cystic tumor. Furthermore, the data presented in Example 2 indicate that 6 out of 18 patients with a variety of tumors (e.g. breast, thyroid, renal cell, lung) showed positive results for tumor vascular response, indicating that "the compound of formula 1 is a highly active agent as manifested by clinical response and acute tumor vascular changes." (see Example 2, page 11 at end of paragraph [0106]).

In addition, Applicants herewith provide several published articles, presentations, and other additional data (see Exhibit 2) that indicate that the compound of formula I (referred to as either AG-013736, or axitinib) may be effective in treating a variety of cancers. In particular, please note the following documents provided in Exhibit 2:

1) Several pages (pp. 16-39) from an internal report summarizing pre-clinical studies of axitinib indicate that this compound showed significant tumor growth inhibition in several xenograft mouse models of a variety of cancers, including colon (see pp. 16-21), breast (see pp. 21-24), lung (see pp. 25-28), melanoma (see pp. 28-32), renal cell carcinoma (see pp. 32-33), glioblastoma (see pp. 33-36), and non-Hodkin's lymphoma (see pp. 36-38).
2) Published journal article (Larkin et al. *Crit. Rev. Oncol*. 60:216-226 (2006)) that describes (p. 220) a Phase II study involving axitinib (5 mg BID) for the treatment of metastatic renal cell carcinoma, wherein 46% of patients showed a partial response.
3) Published abstract (J. Spano et al.) wherein phase I data is reported from a human clinical trial of axitinib in combination with gemcitabine in patients with advanced pancreatic cancer. This study concluded that the combination of axitinib and gemcitabine is safe and effective for pancreatic cancer patients. In particular, significant tumor regression was observed in 2 out of 8 patients.
4) Copies of a set of slides that were used to present data at the 2006 American Society of Clinical Oncology annual meeting. These slides summarize the results of a Phase II clinical study in which patients with thyroid cancer were treated with axitinib (5 mg BID). In particular, partial response was observed in 22% of the patients.

Accordingly, based on the present amendments and the comments and data referred to above, Applicants believe the rejection based on enablement has been overcome and respectfully request that it be withdrawn.

35 U.S.C. §102 (b)

The Examiner also rejects claims 1-30, and 32-47 under 35 U.S.C. §102(b) as allegedly being anticipated by Kania et al. (WO 2001/02369). In particular, the Examiner asserts that Kania discloses a composition containing a compound of formula I, dosing amounts, and methods of

use in treating cancer (see Office Action, pp. 9-11). Applicants respectfully traverse this rejection for the reasons that follow.

A rejection under 35 U.S.C. §102 is only appropriate where the reference discloses each and every limitation of the claim. MPEP §2131. The present claims are directed to specific dosage forms of the compound of formula I, and to methods of treating cancer using these specific dosage forms. Applicants submit that Kania does not disclose the specific dosage forms of the compound of formula I as recited in the present claims.

The Examiner notes that Kania discloses the specific compound of formula I. The Examiner further asserts that Kania discloses a dosage amount of 0.001-50 mg and thus rejects the claims as being anticipated (see Office Action, p. 10). Significantly, the reference to dosing amounts referred to by the Examiner in Kania does not disclose "0.001-50 **mg**" but rather "about 0.001 to about 50 **mg/kg body weight**…" (see Kania U.S. Pat. No. 6,531,491, column 21, lines 31-32, *emphasis added*). Assuming a typical human weight of 80 kg, this range translates into 0.08 mg to 4000 mg. Furthermore, this disclosure in Kania of a broad dosing range is provided in a very generic sense and does not refer specifically to the compound of formula I. Kania discloses a genus of compounds, and provides numerous specific examples of compounds within this genus. The compound of formula I is but one of the numerous examples disclosed. Thus, the disclosure in Kania of a dosing range of 0.001-50 mg/kg applies generically to all compounds, and does not necessarily point to the specific compound of formula I. It is well settled that a generic disclosure does not necessarily anticipate a species within that genus MPEP §2131.02. Thus, although the compound of formula I is disclosed in Kania, there is no disclosure of a ***specific dosage amount*** for the compound of formula I. Accordingly, Applicants assert that Kania does not disclose each and every limitation of any of the present claims as amended.

Furthermore, even if Kania specifically taught the compound of formula 1 in a dosing amount of "0.001-50 mg/kg body weight", it still does not disclose or suggest the specific dosage amount of "no more than 30 mg" (as in claim 8), or any of the other specific dosage amounts in the claims (e.g. 0.5-30 mg, 1-20 mg, 1.5-15 mg, 2-10 mg, 2.5-8 mg, and 3-7 mg). It is well settled that a broad range does not anticipate a specific amount, or a specific smaller range, that is within the broad range, unless the claimed amounts or ranges are disclosed in the prior art with "sufficient specificity". MPEP §2131.03. Applicants assert that a disclosure of "0.001-50 mg/kg body weight" with reference to numerous compounds in Kania does not constitute a disclosure with "sufficient specificity" of "no more than 30 mg" of the ***specific compound of formula I***. Accordingly, Applicants assert that Kania does anticipate any of the present claims.

Finally, the Examiner points to disclosure in Kania that refers to methods of using the compounds disclosed therein for treating various types of cancer. As discussed above, however, this disclosure is provided in a very generic sense with reference to all of the compounds in Kania, and is not specifically directed to the compound of formula I. Furthermore, Kania does not

disclose the use of the specific compound of formula I for treating cancer using the ***specific dosage amounts*** as recited in the present claims.

In view of the above comments, the Examiner has not set forth a proper rejection based on novelty for claims 1-30, and 32-47. Applicants respectfully request that this rejection be withdrawn.

35 U.S.C. § 103

The Examiner also rejects claims 17-48 under 35 U.S.C. §103(a) as allegedly being obvious in view of Kania et al. In particular, the Examiner repeats the same arguments put forth with regard to the rejections based on novelty as discussed above (see Office Action, pp. 12-14). Applicants respectfully traverse this rejection for the following reasons.

To establish a *prima facie* case of obviousness, there must be some suggestion, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. MPEP §2143.01. Additionally, there must be a reasonable expectation of success. MPEP §2143.02.

As noted earlier, the present claims are directed to specific dosage forms of the compound of formula I, and to methods of treating cancer using these specific dosage forms. Applicants submit that neither Kania nor the general knowledge of one skilled in the art teach or suggest the specific dosage forms of the compound of formula I as recited in the present claims.

As discussed above, the compound of formula I is one among numerous specific compounds that are disclosed in Kania. Furthermore, the broad range of dosage amounts (i.e. 0.001-50 mg/kg body weight) mentioned in Kania is not specifically directed to the compound of formula I, but generically refers to all of the compounds disclosed in Kania. In contrast, the present set of claims are directed to very ***specific dosage amounts***, and to methods of treating cancer using very ***specific dosage amounts***, of a ***single particular compound***. Although Kania discloses this specific compound, there is no teaching or suggestion of the ***specific dosing amounts*** for this particular compound that may be useful in treating cancer. Those of skill in the art recognize that the actual dosing amount of a specific compound that is required to successfully treat cancer is highly unpredictable and can only be determined after sufficient clinical testing. For example, the dosing range that will provide therapeutic efficacy in humans, but that will not induce dose limiting toxicities, is not easily predicted without extensive human clinical trials. Kania simply does not teach or suggest the specific dosing amounts for the compound of formula I that are recited in the present claims.

The Examiner also asserts that it would have been obvious to combine the compound of formula I with docetaxel in view of Kania and Sweeney et al. (see Office Action, p. 14). Again, Applicants respectfully traverse this rejection for the following reasons.

Sweeney provides evidence of synergy between a recombinant humanized monoclonal antibody that binds vascular endothelial growth factor (VEGF) and docetaxel as an antiangiogenic therapy. By contrast, the compound of formula I is an inhibitor of a different target (i.e. VEGF-R and PDGFR-β). Furthermore, there is simply no teaching or suggestion in Kania or in Sweeney to use the ***specific dosage amounts*** of the compound of formula I as recited in the present claims. As discussed previously, the specific dosing amount for a particular compound that is safe and effective is highly unpredictable and can only be determined through appropriate clinical testing. Determining an appropriate dosage amount of a particular compound to be used in combination with a second compound likewise requires extensive clinical testing. Even if Kania and Sweeney provide motivation to use the compound of formula I in combination with docetaxel, there is certainly no teaching or suggestion of the ***specific dosage amounts*** as recited in the present claims.

In view of the above, the Examiner has not set forth a *prima facie* case for obviousness for claims 17-48. Applicants respectfully request that this rejection be withdrawn.

Conclusion

Applicants believe all pending claims are now in condition for allowance. Should there be any issues that have not been addressed to the satisfaction of the Examiner, Applicants invite the Examiner to contact the undersigned attorney.

If any fees other than those submitted herewith are due in connection with this response, including the fee for any required extension of time (for which Applicants hereby petition), please charge such fees to Deposit Account No. 16-1445.

Respectfully submitted,

Date: January 24, 2007

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Exhibit 1

Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors

See the related Commentary beginning on page 1277.

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Functions of receptor tyrosine kinases implicated in angiogenesis were pharmacologically impaired in a mouse model of pancreatic islet cancer. An inhibitor targeting VEGFRs in endothelial cells (SU5416) is effective against early-stage angiogenic lesions, but not large, well-vascularized tumors. In contrast, a kinase inhibitor incorporating selectivity for PDGFRs (SU6668) is shown to block further growth of end-stage tumors, eliciting detachment of pericytes and disruption of tumor vascularity. Importantly, PDGFRs were expressed only in perivascular cells of this tumor type, suggesting that PDGFR$^+$ pericytes in tumors present a complimentary target to endothelial cells for efficacious antiangiogenic therapy. Therapeutic regimes combining the two kinase inhibitors (SU5416 and SU6668) were more efficacious against all stages of islet carcinogenesis than either single agent. Combination of the VEGFR inhibitor with another distinctive kinase inhibitor targeting PDGFR activity (Gleevec) was also able to regress late-stage tumors. Thus, combinatorial targeting of receptor tyrosine kinases shows promise for treating multiple stages in tumorigenesis, most notably the often-intractable late-stage solid tumor.

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Introduction

Neovascularization is a common attribute of tumors, and a wealth of functional studies support the proposition that blood vessels are crucial for the formation, growth, and dissemination of cancer (1, 2). Animal models of cancer, including both traditional tumor transplants and newer genetically engineered mouse models of cancer, have helped establish the causality of angiogenesis and presented platforms for assessing antiangiogenic therapeutic strategies (3, 4). The latter have further revealed that the normally quiescent tissue vasculature is characteristically first activated by an "angiogenic switch" to produce new blood vessels during hyperproliferative premalignant phases of carcinogenesis, before solid tumors have formed (5–7). One such model, the RIP1Tag2 line of transgenic mice, has been particularly instructive about parameters of angiogenesis and the prospects for antiangiogenic therapy. By virtue of expressing the SV40 virus oncoproteins in the pancreatic islet β cells, RIP1Tag2 mice develop islet carcinomas in a multistep pathway characterized by the temporal appearance of distinctive lesional stages: hyperplastic/dysplastic islets (with quiescent vasculature); angiogenic dysplastic islets; solid tumors with well-defined margins and fibrous capsules; and invasive carcinomas (8–10). The focal nature of the approximately 400 islets in the mouse pancreas and the relative synchronicity of progressive appearance of these lesions served to reveal the angiogenic switch as a discrete step in carcinogenesis (5). Furthermore, this model has afforded the design of preclinical therapeutic trials based on the distinctive stages of tumor development (3). In an assessment of four candidate angiogenesis inhibitors, differential stage-specific efficacy was observed: three agents (the protease inhibitor BB94/batimastat, endostatin, and angiostatin) performed best in treating early stage disease, both in the prevention trial targeting angiogenic switching in dysplastic lesions and in the mid-stage intervention trial aimed at blocking the expansive growth of small, solid tumors. Another inhibitor (TNP470) was effective at reducing the mass of bulky end-stage tumors in a regression trial, but it did not perform well in the early-stage prevention trial. These differential responses to antiangiogenic drugs suggested that there might be qualitative differences in the angiogenic vasculature in early and late stages or in the

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Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: receptor tyrosine kinase inhibitor (RTKI); paraformaldehyde (PFA); FGF receptor (FGFR); platelet-endothelial cell adhesion molecule (PECAM).

regulatory mechanisms that control induction of angiogenesis and persistence of the tumor vasculature. This concept of stage-specific efficacy has been strengthened by recent studies investigating the effects of a kinase inhibitor SU5416 (11) that selectively inhibits the VEGFRs controlling angiogenic activity of endothelial cells (12, 13).

Both pharmacological inhibitors and gene knockout approaches have been used to investigate the means by which the angiogenic switch is activated and sustained in this model. Key components of the switching mechanisms have proved to be a matrix protease, MMP-9, which mobilizes an angiogenic factor, VEGF-A, that in turn binds to a receptor tyrosine kinase expressed on endothelial cells, VEGFR2 (12, 14). Abrogation of MMP-9 by gene knockout or pharmacological inhibition reduced the frequency of angiogenic switching and impaired tumor growth (12). Furthermore, pharmacological inhibition of VEGF signaling (12) or targeted deletion of the *VEGF* gene (14) almost completely blocked the angiogenic switch in premalignant lesions and severely impaired growth of small tumors. The few tumors that developed in RIP1-Tag2 mice whose islets lacked VEGF, were small, avascular, and necrotic, without any features of neovascularization. These studies demonstrated the importance of VEGF-signaling for angiogenic switching, tumor formation, and initial tumor growth in this model. Remarkably, however, we have reported recently (12) that inhibition of VEGFR signaling, either indirectly with a MMPI, or directly with a VEGFRI (SU5416), was not efficacious in a regression trial against late-stage islet tumors, which continued to grow. Combination of either class of inhibitor with an antiangiogenic, "metronomic" chemotherapy regimen (12, 15, 16) produced stable disease or modest regression of such tumors (13), encouraging the proposition that combinatorial targeted therapies might be a key to achieving late-stage efficacy with a VEGFR inhibitor. To that end we have used the RIP1Tag2 mouse model to investigate the stage specific efficacy profile of a receptor tyrosine kinase inhibitor (RTKI) with broader specificity and have assessed the benefits of combination strategies involving distinctive RTKIs. The studies reported below encourage multiplex receptor-targeting strategies and have in particular highlighted the potential significance of PDGFR signaling in tumor-associated pericytes, thereby implicating this cell type as a functionally important component of the tumor vasculature and a new target for antiangiogenic therapy.

Methods

Drug treatment of transgenic mice. The mice in these studies were males and females of the RIP1Tag2 transgenic mouse lineage that were bred 45 generations into the C57Bl/6J background. Animals were treated from 5 to 10.5 weeks of age in the prevention trial, from 10 to 13.5 weeks in the intervention trial, and from 12 to 16 weeks in the regression trial. All control mice received subcutaneous or oral saline injections. SU6668 and SU5416 (Sugen Inc., South San Francisco, California, USA) were provided in vehicle formulations. As single agents, 200 mg/kg of SU6668 was administered orally every day, and 100 mg/kg SU5416 was inoculated subcutaneously twice a week; if combined with other drugs, the SU5416 dose had to be reduced to 50–75 mg/kg. Studies were limited by toxicities (weight loss, lung hemorrhages) associated with SU5416, predominantly in end-stage mice (older than 14 weeks), which appear to relate to the chemistry of SU5416 rather than its mechanism of action. We have not, for example, observed such toxic side effects in trials with another VEGFR inhibitor (O. Casanovas and D. Hanahan, unpublished observations). Gleevec/STI57 (50 mg/kg; Novartis Pharma AG, Basel, Switzerland) was administered orally twice a day (17). All trials were repeated up to three times. Mice were maintained in accordance with the University of California, San Francisco (UCSF) institutional guidelines governing the care of laboratory mice, and euthanized after the respective treatment period or when tumor burden and/or side effects obligated their removal from study.

Assessment of the angiogenic islets and tumor burden. In the prevention trial, angiogenic islets were isolated by retrograde perfusion with collagenase solution and counted. Angiogenic islets were identified as those that exhibited a reddish patch or patches (caused by hemorrhaging) in a white nodular background (18). In the intervention and regression trials, animals were euthanized at the end of the respective trial and tumors microdissected from freshly excised pancreata. Tumor volume (cubic millimeters) was measured by using a caliper, applying the formula [volume = $0.52 \times (\text{width})^2 \times (\text{length})$] for approximating the volume of a spheroid. Tumor burden per mouse was calculated by accumulating the tumor volume of every mouse.

Visualization of the vasculature. To visualize blood vessels in tumors and normal tissue, mice were first anesthetized and injected intravenously with 0.05 mg FITC-labeled tomato lectin (*Lycopersicon esculentum*; Vector Laboratories, Burlingame, California, USA), then the heart was perfused with 4% paraformaldehyde (PFA). Pancreata were frozen in OCT medium and sectioned at 50 μm.

Immunohistochemical analysis. Mice were anesthetized, hearts perfused with PFA, and pancreata collected, frozen in OCT medium, and 15-μm sections prepared. Pericytes were identified with a mouse anti-human desmin Ab (1:3,000; DAKO Corp., Carpinteria, California, USA), a marker of mature pericytes, and endothelial cells were detected with a rat anti-mouse CD31 Ab (1:100, BD PharMingen, San Diego, California, USA). To reveal the Ab reactions, sections were then incubated with either a CY3-labeled goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA), or a FITC-labeled goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories Inc.), or a rhodamine-labeled goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories Inc.). To visualize endothelial and perivascular cells, sections were either simultaneously stained with a CD31 and desmin

Ab, or immunohistochemistry was performed with the desmin Ab on cryosections of mice whose vascular system had been perfused with lectin-FITC before euthanasia. PDGFR-β^+ cells were deleted with a rat-autoimmune PDGFR-β antibody from eBioscience (San Diego, California, USA).

FACS analysis. Mice were sacrificed and tumors excised from the pancreas and minced with a razor blade on ice in 1× PBS. The minced tumor fragments were then digested at 37°C for 13 min with a collagenase mix containing 0.2 g BSA (Sigma Aldrich, St. Louis, Missouri, USA), 0.05 g collagenase II, 0.05 g collagenase IV, 0.02g DNase I (all from Worthington Biochemical Corp., Lakewood, New Jersey, USA), and passed through a 70-μm cell strainer. The cells were washed, the red blood cells lysed with PharM Lyse (BD PharMingen) for 15 sec, and then washed again. The cell pellets were resuspended in FACS buffer (1× PBS plus 1% BSA), preblocked with an Fc block (CD16/CD32; BD PharMingen), and then incubated with primary Ab on ice: CD31-PE, 1:100; Ly-6G, 1:60 (recognizing the GR-1 antigen on granulocytes), from BD PharMingen; CD11b, 1:40 (recognizing the Mac-1 antigen on macrophages) and other immune cell types from BD PharMingen. The cells were washed and Via-Probe was added as a cell death indicator (BD PharMingen). The cells were then sorted on a FACSVantage SE flow cytometer using the Cell Quest Pro software version 4 from Becton Dickinson Immunocytometry Systems (Franklin Lakes, New Jersey, USA). The FL2 gate identified the $CD31^+$ cells, the FL1 gate identified the $Gr\text{-}1^+/Mac\text{-}1^+$ cells, and the FL3 gate excluded the dead cells.

RNA isolation and RT-PCR analysis. FACS-sorted cells were collected in a cell lysis solution from QIAGEN Inc. (Valencia, California, USA) containing β-mercaptoethanol. RNA was isolated following RNeasy Mini Kit protocols (QIAGEN Inc.) and transcribed into single-stranded cDNA using Superscript II RNase H^- Reverse Transcriptase (Invitrogen Corp., Carlsbad, California, USA). RT-PCR analysis was performed using custom primers (QIAGEN Inc.) for PDGF-A, -B, -C, -D, PDGFR-α and -β, desmin, and smooth-muscle actin, and for L19 as internal control.

Results

Two RTKIs have distinctive efficacy profiles. Previously, we evaluated the VEGFR inhibitor SU5416 (11, 19) in the three distinctive therapeutic trials in RIPTag2 mice (12, 13); these data are illustrated here to facilitate evaluation of the comparative and combination trials involving this and other RTKI. In the regression trial, which treats end-stage mice having bulky disease, thus being analogous to the typical phase-3 clinical trial of investigational anticancer drugs (Figure 1), treatment with SU5416 produced a modest increase in life span (to a defined endpoint 2.5 weeks after sham-treated mice were euthanized due to incipient death from tumor burden), concomitant with a lower rate of tumor growth, but this drug was not capable of regressing tumor mass or producing stable disease (Figure 1; RT). As such, we found the SU5416 efficacy profile to be similar to that of endostatin, angiostatin, and the MMP inhibitors BB94 and BAY-129566 (3, 12, 13).

Given that SU5416 was very efficacious against earlier-stage disease (Figure 1), phenocopying the *VEGF-A* gene knockout in its impairment of angiogenic switching and tumor growth, we reasoned that other regulatory molecules might become involved in controlling angiogenesis and maintaining of the tumor vasculature in well-established solid tumors. We therefore evaluated another RTKI (SU6668) with somewhat broader selectivity in the three distinctive preclinical trials in the Rip1Tag2 model.

SU6668, a small molecule kinase inhibitor with demonstrable antiangiogenic activity (20–22), inhibits phosphorylation and signal transduction of PDGFRs, VEGFRs, and FGF receptors (FGFRs). While SU6668 has significantly higher biochemical activity against PDGFR-α and -β (K_I = 0.0008 μM) than VEGFR-2 and FGFR-1 (K_I = 2.1 μM and 1.2 μM, respectively) (21), cell-based assays reveal functionally appreciable inhibitory

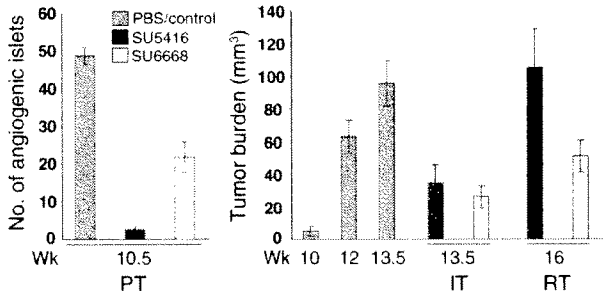

Figure 1
Different stage-specific efficacy profiles for the VEGFR inhibitor SU5416 and the PDGF (+VEGF/FGF) receptor inhibitor SU6668 in the three distinct stages of pancreatic islet carcinogenesis in RIP1Tag2 transgenic mice. Mice were either treated with SU5416 or SU6668 as described in Methods. The average number of angiogenic islets ± SEM at 10.5 weeks in control and treated mice, the average tumor burden ± SEM in PBS/vehicle-treated mice (at 10, 12, 13.5 weeks), and SU5416- and SU6668-treated mice (at 13.5 and 16 weeks) are shown. The prevention trial (PT) started at 5 weeks, when mice harbor hyperplastic/dysplastic islets, and ended at 10.5 weeks, when the first small tumors appear. Islets that have switched on angiogenesis are scored by their reddish color (resulting from microhemorrhage and leakiness associated with VEGF-induced angiogenesis). In the intervention trial (IT), mice with a small tumor burden (10 weeks) are treated until the end stage (13.5 weeks), while in the regression trial (RT), 12-week-old mice with substantial tumor burden and a life expectancy of less than 2 weeks are treated until 16 weeks, when control mice are already dead. Statistical analysis was performed with a two-tailed, unpaired Mann-Whitney test comparing experimental groups to PBS-injected control mice. Tumor burdens of experimental groups in the Regression Trial were compared to that of 12-week-old Rip1Tag2 mice. Cohorts of 6–21 animals were used. *P* values less than 0.1 are considered statistically significant. *P* values of SU5416 PT = 2.26×10^{-5}, SU6668 PT = 0.0002, SU5416 IT = 0.0009, SU6668 IT = 0.0001, SU5416 RT = 0.1827, and SU6668 RT = 0.3228.

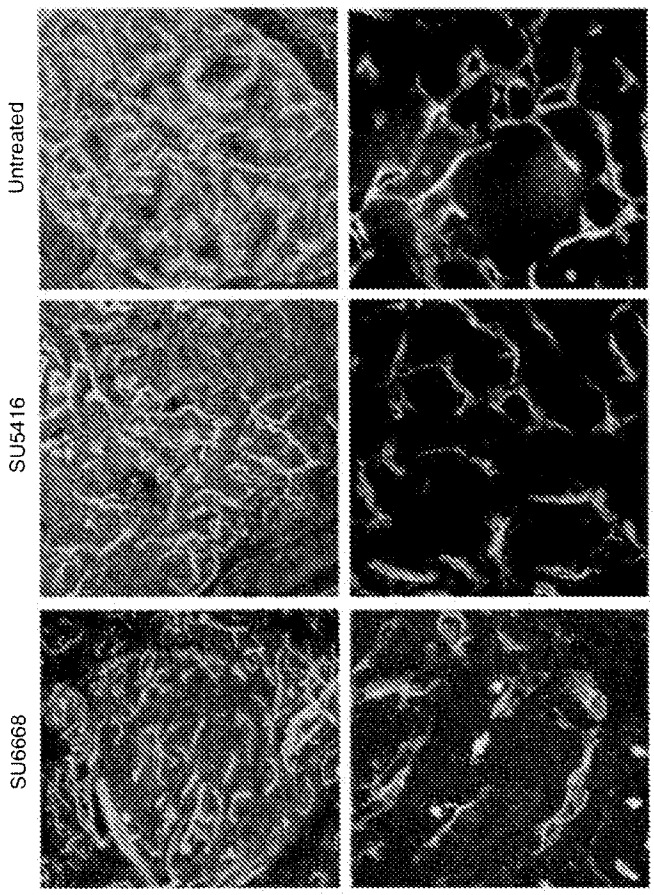

Figure 2
Comparison of vascular morphology (left panels) and association of endothelial cells and perivascular cells (right panels) in treated versus control tumors. Tumor-bearing pancreata were taken from end-stage 13.5-week-old control Rip1Tag2 mice and from 16-week-old Rip1Tag2 mice treated with SU5416 or SU6668 (Regression Trial). To visualize the functional blood vessels in tumors, mice were first anesthetized and injected intravenously with 0.05 mg FITC-labeled tomato lectin (*Lycopersicon esculentum*) and then heart perfused with 4% PFA. Pancreata were frozen in OCT medium and sectioned at 50 μm. To visualize endothelial cells (green in right panels) and pericytes (red in right panels) by immunohistochemical analysis, mice were anesthetized, heart perfused with PFA, and pancreata collected, frozen in OCT medium, and 15-μm sections prepared. Endothelial cells were detected with FITC-labeled lectin; pericytes were identified with CY3-labeled anti-desmin (1:3,000), a marker of mature pericytes.

activity against both VEGFR-21 and PDGFR (21, 23, 24). By contrast, SU5416 is predominantly active against VEGFR-2 (K_I = 0.04 μM), with minimal activity against PDGFR or FGFR (11, 25).

The trials with SU6668 produced a different efficacy profile from that of SU5416. Thus, SU6668 was less effective at blocking angiogenic switching in the prevention trial and was similar at impairing tumor growth in an intervention trial (Figure 1). Surprisingly, this agent proved much more effective than SU5416 in the regression trial, producing a condition of stable disease, in that tumor burden at the culmination (a defined end point after 4 weeks of treatment) was similar to that at the 12-week-old starting point. How can these differences be explained? We suspect that the relatively poor activity of SU6668 at blocking angiogenic switching (Figure 1; PT) reflects its lower K_I against VEGFR; by contrast, SU5416 produced similar reductions in angiogenic switching to the gene knockout of *VEGF-A* (14), indicative of the singular importance of VEGF signaling at this early stage. As for its significantly better effects on well-established tumors, we hypothesized that the broader specificity of SU6668, in particular against PDGFR, might underlay its benefits. We sought, therefore, to compare the histological effects of treatment with these two agents that might reflect distinctive functional effects and to investigate the expression of PDGF ligands and receptors in the islet tumors, given that SU6668 potently inhibits these receptors.

To investigate the underlying mechanisms of these stage-specific effects, we first assessed the vascular morphology in the treated versus control tumors from the regression trials of these two drugs. Perfusion of the circulatory system with a fluorescent lectin was used to assess the functional tumor vasculature, revealing (Figure 2; left panel) demonstrable decrease in vascularity of SU6668-treated tumors and modest reduction in tumors treated with SU5416. We further assessed the vascular morphology by immunostaining tissue sections with Ab's recognizing two vascular markers: CD31 (PECAM), a cell adhesion molecule expressed on endothelial (and hematopoietic) cells, and desmin, a marker of mature periendothelial support cells (pericytes) (26, 27). We were motivated to investigate pericytes both by a report (28) and our unpublished data that pericytes were abundant in tumors of the RIP1Tag2 model. A typical intimate association was seen between endothelial cells and pericytes in the untreated tumors, as well as in the SU5416-treated tumors (Figure 2; right panel). By contrast, SU6668-treated tumors showed marked disruption of pericyte-endothelial cell association. Pericytes had become separated from the endothelial cells, and the blood vessels were enlarged and distorted (Figure 2). Thus, SU6668 affected the tumor vasculature differently than SU5416: not only was the vascularity of the tumors reduced, but the integrity of the association of endothelial cells and pericytes was markedly perturbed. Collectively, the results led us to suspect that the distinctive effects of SU6668 against solid tumors resulted from its targeting of pericytes; this was further supported by a report suggesting that SU6668 could reduce the number pericytes in a tumor transplant model (29). Further rationale for this hypothesis came from the knowledge that pericytes express PDGFRs during vessel formation in the developing embryo and that functional disruption of PDGFR-β or its ligand

PDGF-B leads to a lack of pericytes, causing severe vascular defects and embryonic lethality in late gestation (30–32). Such considerations raised a question: were PDGFRs expressed in pericytes or other cell types in the islet tumors, and if so were PDGF ligands expressed?

Expression in tumor vasculature of PDGF ligands and PDGFRs. To assess the expression of PDGF ligands and PDGFRs, RNA was isolated from whole tumors and analyzed by RT-PCR, revealing expression of the PDGF-A, -B, and -D ligands, as well as both PDGFR-α and -β (Figure 3a). To identify the cell type that expressed PDGF ligands and receptors, we developed a protocol for fractionating primary tumors into constituent cell types by flow cytometry. Endothelial cells were sorted as $CD31^+$, $Gr1^-$, $Mac1^-$; inflammatory cells were collected as $Gr1^+$, $Mac1^+$ (not shown); and tumor cells were gated by size and collected as unlabeled with these three Ab's. RNA was collected from unsorted and sorted populations and analyzed by RT-PCR. As illustrated in Figure 3a, the endothelial cells were found to exclusively express the genes for PDGF ligands A, B, and D. None of the sorted cell populations, tumor cells, inflammatory cells (not shown), or endothelial cells, expressed the two PDGFR genes, despite demonstrable expression in whole-tumor RNA. We therefore stained tumor tissue sections with Ab's specific for PDGFR-β to ask whether the receptor was expressed, and if so, in which cell type. The tissue sections were costained with anti-CD31/PECAM to mark the endothelial cells. The data shown in Figure 3b clearly reveal expression of PDGFR-β in perivascular cells that are in close contact with endothelial cells, but not in the endothelial cells or tumor cells, consistent with the analysis of the sorted cell types. (PDGFR-α expression is very low in the pancreatic tumors, and the mouse protein could not be detected with available Ab's in tissue sections.) We went on to collect the $PDGFR^+$ cells by FACS using an anti–PDGFR-β Ab; this cell population proved to have a small size that was gated out in the unlabeled tumor cell fraction shown in Figure 3a and furthermore expressed known markers of pericytes (e.g., desmin and smooth-muscle actin [SMA], as shown in Figure 3c) consistent with identification of the PDGFR-β^+ cells in these tumors as a class of pericyte. The data indicate that PDGF ligands are expressed in the tumor endothelial cells and that PDGFR-β is expressed in cells associated with the angiogenic vasculature that morphologically score as perivascular cells (pericytes). The data are consistent with the hypothesis that SU6668 is targeting $PDGFR^+$ pericytes, causing their dissociation from the tumor vasculature, leading to vascular dysfunction. It is formally possible that the VEGFR activity of SU6668 is contributing to the observed effects on tumor growth and tumor vascularity. The VEGFR selective inhibitor SU5416 did not induce dissociation of pericytes from tumor blood vessels (Figure 2), however, suggesting that SU6668's modest activity against the VEGFRs is not the primary basis for its effect on tumor pericytes. We cannot at present exclude possible contributions to the observed effects of SU6668's inhibition of FGFRs, given that there is evidence supporting involvement of FGF signaling in this model (33). Data to be presented below, however, suggest that the primary activity of SU6668 in this model of pancreatic islet carcinogenesis involves its inhibition of PDGFR signaling.

Improved efficacy by combining kinase inhibitors with distinct specificity. SU5416 was most efficacious at blocking initial angiogenic switching and similarly as effective as SU6668 at repressing growth of small, nascent, solid tumors, whereas SU6668 was more effective against end-stage bulky disease (Figure 1). Therefore, we reasoned that combining the two kinase inhibitors might improve efficacy, given their distinctive efficacy profiles and target selectiveness. To investigate this hypothesis, combination trials were performed. The data are provocative. The combination of SU5416 and SU6668 improved efficacy in each of the three trials,

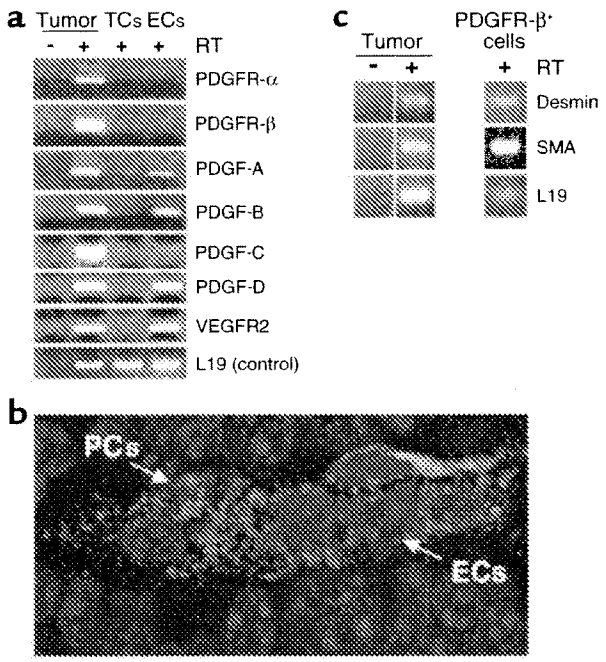

Figure 3

Identification of the cell types expressing PDGF ligands and receptors in pancreatic islet carcinomas. (**a**) Primary tumors were fractioned into constituent cell types by flow cytometry. RNA was isolated from unsorted and sorted populations and analyzed by RT-PCR. Pancreatic tumors of end-stage Rip1Tag2 mice were excised and enzymatically dispersed with collagenase into single cells. The cell suspension was incubated with Ab's for CD31 and Gr1 and Mac1. Endothelial cells were collected by FACS as a $CD31^+$, $Gr1^-$, $Mac1^-$ population, whereas tumor cells were gated by size and collected as unlabeled with these three Ab's. Inflammatory cells were collected as $Gr1^+$, $Mac1^+$; these cells did not express PDGF ligands or receptors (not shown). (**b**) Tumor sections (prepared as in Figure 2) were costained with anti–PDGFR-β-FITC (1:200) and anti-CD31-rhodamine to reveal PDGFR-β-expressing cells in green and endothelial cells in red. (**c**) PDGFR-β^+ cells from tumors were isolated by FACS (PDGFR-β Ab, 1:50), RNA isolated, and analyzed by RT-PCR for pericyte markers. ECs, endothelial cells; TCs, tumor cells; PCs, perivascular cells.

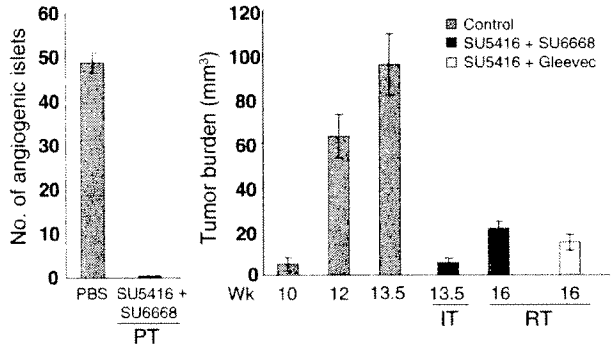

Figure 4

Improved efficacy at all stages of islet carcinogenesis produced by combining the VEGFR-inhibitor SU5416 with SU6668 or Gleevec, two drugs that inhibit PDGFR signaling. Mice were injected subcutaneously with 50–75 mg/kg SU5416 twice a week and in addition received either daily oral administration of 200 mg/kg SU6668 or twice daily dosing of 50 mg/kg Gleevec (STI571). (The dosage of SU5416 had to be reduced from that used in the single-agent trials shown in Figure 1 due to SU5416-specific toxic side effects.) The average number of angiogenic islets ± SEM at 10.5 weeks in control and treated mice are shown in the prevention trial. The average tumor burden ± SEM of PBS/vehicle-treated mice is indicated at 10, 12, and 13.5 weeks, for comparison with SU5416 + SU6668-treated mice at 13.5 and 16 weeks, and with SU5416 + Gleevec-treated mice at 16 weeks. Tumor burdens were assessed as described in Methods. Statistical analysis was performed with a two-tailed, unpaired Mann-Whitney test comparing experimental groups with PBS-injected control mice. Tumor burdens of experimental groups in the regression trial were compared with that of 12-week-old Rip1Tag2 mice. Cohorts of 6–21 animals were used. *P* values less than 0.1 are considered statistically significant. (*P* values of SU5416 + SU6668 PT = 0.0002, SU5416 + SU6668 IT = 0.0008, SU5416 + SU6668 RT = 0.0003, and SU5416 + Gleevec RT = 0.0007.

variously targeting angiogenic dysplasias, small tumors, or end-stage tumors (Figure 4). Thus, the combination was better than either of the single agents at all stages of carcinogenesis, producing a broad efficacy profile. Mice treated with the combination did not develop morphologically identifiable angiogenic islets in the prevention trial, in contrast with the control mice. Histological analysis revealed that treated islets did not have hemorrhages and appeared more benign, like early hyperplastic islets in which the cells had a larger cytoplasm-to-nucleus ratio (Figure 5, a and b). Most exciting was the clear and convincing reduction in tumor mass seen in the regression trial. Not only did the combination produce severe reductions in tumor mass (Figures 4 and 5, c and d), but it also elicited evident necrosis and limited the characteristic microhemorrhaging that produces blood-filled tumors (Figure 5, e and f). The blood vessel network regressed and predominantly disappeared in larger tumors, leaving the perivascular cells stretched out and distorted (Figure 5, g and h). Concomitantly, apoptosis was increased 6.7-fold (Figure 5, i and j) in the combination therapy, whereas single treatment with SU5416 or SU6668 increased apoptosis 2.7- and 3.5-fold, respectively. The data presented above suggested that these dramatic effects are the result of simultaneous targeting of VEGFRs in endothelial cells and PDGFRs in perivascular cells. The modest inhibitory profile of SU6668 against FGFRs and its demonstrable VEGFR activity, however, cannot absolutely exclude other interpretations. Therefore, we tested another tyrosine kinase inhibitor, Gleevec (STI571), which has high activity against three kinases: Bcr/Abl, c-Kit, and PDGFR (17, 34, 35). Among known kinases tested, Gleevec overlaps with SU6668 only in its inhibition of PDGFR. We performed a regression trial with Gleevec, treating near end-stage mice with Gleevec alone or in combination with SU5416. Gleevec, which has very poor pharmacokinetic properties in mice (R. van Etten, Harvard Medical School, Boston, Massachusetts, USA, personal communication; see ref. 34), was not effectual as a single agent (not shown). Nevertheless, the combination of Gleevec and SU5416 produced significant reductions in tumor burden (Figure 4) comparable to SU6668 plus SU5416 treatment. Furthermore, the Gleevec combination produced similar morphological disruptions in tumors (increased apoptosis, decreased abundance and detachment of pericytes, and reduced vascularity; Figure 5l). Interestingly, the degree of vascular disruption varied among independent tumors, apparently as a function of the size of tumors, being most apparent in larger tumors. Importantly, neither of the combinatorial treatments caused dissociation of perivascular cells from blood vessels of normal tissue (lung, liver; data not shown) or otherwise disrupted normal tissue vasculature, including that of the immediately adjacent exocrine pancreas to the islet tumors (shown for Gleevec plus SU5416 in Figure 5k), indicating that the tumor vasculature is differentially sensitive to these kinase inhibitors.

The dramatic effect both kinase inhibitor combinations had on tumors in a month-long trial raised the question of whether they could improve survival over a longer time period. We sought to treat end-stage mice in a multimonth trial, but found that SU5416 was poorly tolerated by older mice, requiring most mice to be removed from study (see Methods). These side effects precluded statistically significant survival studies. Nevertheless, a few mice were able to stay in trial for 2 months with SU6668 plus SU5416 in the regression trial (i.e., 6 weeks past the incipient death and obligate euthanasia of the untreated controls); these mice still had a smaller tumor burden than mice at the starting point of the trial (12 weeks) (data not shown), indicating that the combination therapies were not only regressing well-established tumors in end-stage mice, but also limiting subsequent regrowth of these tumors or other new tumors forming from the abundant angiogenic progenitors characteristic of this challenging multifocal model of carcinogenesis. We hope to address long-term survival in future studies using less toxic VEGFR inhibitors in conjunction with Gleevec and SU6668, as well as other PDGFR inhibitors.

Discussion

In the course of investigating the efficacy of RTKIs in a mouse model of multistage carcinogenesis, we have made an unexpected observation that may have important implications for therapeutic strategies targeting angiogenesis and the tumor vasculature for the treatment of human cancers. The data support the proposition that perivascular cells associated with the tumor vasculature expressing the PDGFR-β are functionally important for maintenance of tumor blood vessels, adding another constituent cell type in tumors to the list of anticancer targets (Figure 6). Association of PDGFR$^+$ perivascular cells with the tumor endothelial cells is ostensibly maintained by the expression of PDGF ligands in the endothelial cells, establishing a paracrine homeostatic signaling circuit analogous to the situation during embryonic development of certain tissue vascular beds (27, 31, 36). Notably, neither PDGF ligands or PDGFRs are expressed by the tumor cells in this model of pancreatic islet carcinogenesis, unlike many of the tumor transplant models studied with the kinase inhibitors under consideration (20, 29). The lack of tumor cell expression points to the importance of PDGF signaling in perivascular cells for sustaining the tumor vasculature via association with endothelial cells.

There are several reasons to suspect that the actions and interactions of pericytes and endothelial cells in these prototypical tumors are qualitatively different from that in normal tissues. First, the SU6668 single treatment, as well as the combined treatment of SU5416 plus SU6668 or Gleevec, which disrupts the association of pericytes and reduces the vascularity in tumors, has no such effect in normal tissues of the treated mice. This is in agreement with the finding that perturbation of PDGF signaling in the developing retina elicits detachment of pericytes on immature vessels, but not mature vessels, indicating the pericyte-endothelial interactions in newly formed vessels is critically dependent on PDGF (24, 32, 37). Second, blockage of VEGFR also disrupts the tumor vasculature but

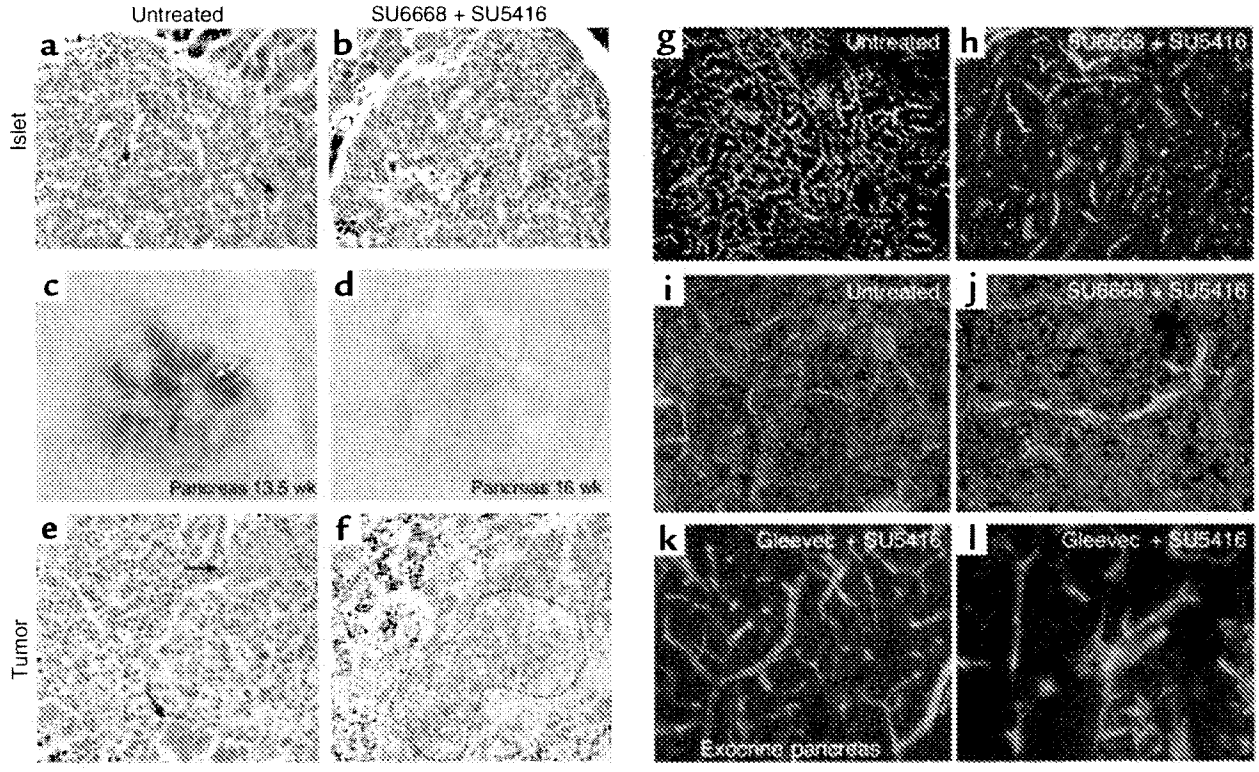

Figure 5

Effects of the combined therapy using SU5416 + SU6668 or SU5416 + Gleevec. Hematoxylin and eosin staining of islets from untreated (**a**) and SU6668 + SU5416-treated transgenic mice (**b**) at 10.5 weeks in a prevention trial. Gross pathology of dissected pancreata from untreated (**c**) and SU5416 + SU6668-treated mice (**d**) in a 4-week regression trial targeting late-stage disease. Hematoxylin and eosin staining of tumors from untreated (**e**) and SU5416 + SU6668-treated mice (**f**). Arrows indicate hemorrhage formation, and dotted area confines necrotic region. Comparison of the functional vasculature in control (**g**) and SU5416 + SU6668-treated mice (**h**) from a regression trial. Mice were injected intravenously with FITC-labeled tomato lectin (*Lycopersicon esculentum*) to stain blood vessels in green, and then heart perfused with 4% PFA, followed by immunohistochemical staining with Cy3-labeled desmin Ab to label desmin-expressing perivascular cells in red. Apoptotic cells in tumors of control (**i**) and SU6668 + SU5416-treated mice (**j**) were detected by TUNEL staining with fluorescent visualization (red), and the vasculature was revealed as above by intravenous FITC-lectin perfusion before sacrifice. Mice were treated with SU5416 + Gleevec in the regression trial, and blood vessels and perivascular cells of exocrine pancreas (**k**) and adjacent islet tumors (**l**) were visualized with FITC-lectin and a Cy3-labeled desmin Ab.

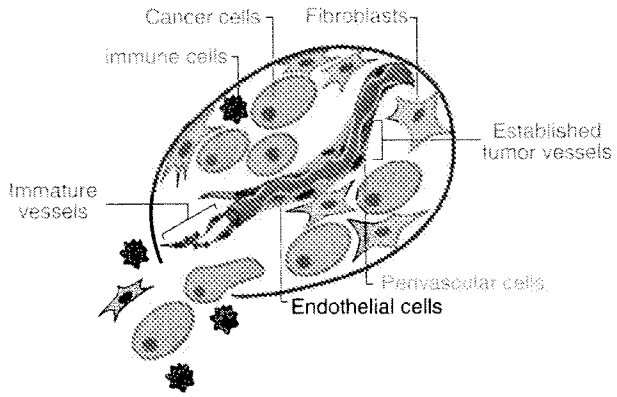

Figure 6
Two distinctive vascular cell types in tumors present complementary targets for anticancer drugs. The results presented herein suggest that the conceptual notion of tumors as aberrant organs composed of both cancer cells and conscripted normal cell types, all making functional contributions to tumor phenotypes (43), be expanded both to include PDGFR-β$^+$ pericytes and to recognize that tumors can have vasculature that is either immature or mature, with different responses to angiogenesis inhibitors. Combined therapy efficacy against otherwise intractable late-stage islet carcinomas is observed when VEGFRs on endothelial cells and PDGFRs on perivascular cells are targeted together. There is reason to envision that when the other constituent cell types, in particular the overt cancer cells, are also targeted, long-term therapeutic benefit can be achieved.

not normal tissue vessels, again revealing different sensitivity. Its effect on tumors was markedly different, however: treatment with the VEGFR-selective inhibitor SU5416 had no impact on pericyte-endothelial association in islet tumors (see Figure 2) despite its clear impairment of angiogenesis. Taken together, these observations suggest that the endothelial cells and pericytes in tumors are abnormal in their regulation and likely their functionality, providing a rationale for the "therapeutic window" we observe in the ability of these agents to selectively disrupt tumor vasculature and effect tumor regression. The basis of the apparent differences in normal and tumor pericytes and endothelial cells and in their paracrine homeostatic signaling deserves future investigation. It is of interest that PDGF signaling recently has been implicated in maintenance of interstitial pressure in tumors, in that inhibitors of PDGFR (including Gleevec) reduced pressure in subcutaneous transplant tumors (38); perhaps impaired pericyte interactions with the tumor vasculature is contributing to this effect.

The use of distinctive preclinical trials targeting different stages in the development of islet carcinomas in the RIP1Tag2 mouse model has revealed a stage-specific efficacy for the VEGFR inhibitor SU5416, which is similar to that of protease inhibitor BB-94, which targets the VEGF-A–activating protease MMP-9 (3, 12, 13). Both investigational drugs were effective in treating premalignant lesions (angiogenic dysplasias) and small tumors, but neither was able to produce responses against late-stage tumors. The lack of efficacy against large tumors is congruent with the failure of three MMPIs in phase-3 clinical trials against late-stage human cancers (39, 40) and predicts the same for VEGFR inhibitors: notably, SU5416 was recently withdrawn from phase-3 clinical trials against late-stage cancers, perhaps reflecting a similar lack of efficacy (http://www.sugen.com/webpage_templates/sec.php3?page_name=pr_1013191807&press_release=1&year = 2002). By contrast, SU6668 had a distinctive efficacy profile in the stage-specific trials in the RIPTag2 model: it was less effective in the prevention trial targeting angiogenic dysplasias, but was much better in the regression trial, producing a condition of "stable disease." Remarkably, the combination of the two RTKIs produced clear and convincing responses in all three trials, which in every case was better than either single agent alone. The synergy supports the arguments above that these agents are targeting different signaling circuits and, indeed, distinct vascular cell types in tumors. The proposition that SU6668 is predominantly an inhibitor of PDGFR signaling is supported by the combinatorial trials using SU5416 plus Gleevec, which inhibits PDGFR along with Bcr/Abl and c-Kit, but not FGFR or VEGFR. Thus, SU6668 and Gleevec share activity only against PDGFR, among the known kinases tested. Certainly highly specific inhibitors that pharmacologically knock out only PDGFR signaling will be necessary to unequivocally prove the concept. Toward that end, in a recent pilot study treating RIPTag2 mice with an adenovirus expressing a soluble form of PDGFR, we observed pericyte detachment from vessels in pancreatic islet tumors but not normal adjacent exocrine pancreas, supporting the hypothesis that PDGFR signaling is critical for pericyte-endothelial association in tumors and is a basis for the effects seen with SU6668 and Gleevec (G. Bergers, unpublished observation).

Not only is the objective response seen with the regimen of Gleevec plus SU5416 conceptually significant, it could be important clinically. Gleevec has been approved for clinical practice by the FDA, and thus it is applicable to a standard protocol for testing investigational drugs, namely combining such candidates with an approved drug. We suggest that Gleevec will show efficacy against human tumors when supplied in combination with an inhibitor of VEGF signaling. Given that a number of drugs aimed at capturing the VEGF-A ligand or inhibiting the VEGFR kinase are in clinical trials, this is a testable hypothesis. Importantly, it should not be necessary that the tumor cells express the kinases that Gleevec targets. Rather, the data presented above suggest that Gleevec (as well as SU6668 and other PDGFR inhibitors) will synergize with inhibitors of VEGF signaling targeting endothelial cells by inhibiting PDGFR signaling in periendothelial cells, thereby targeting interdependent cellular constituents of the tumor vasculature, consequently rendering antiangiogenic therapies more broadly efficacious. This proposition could be tested clinically with Avastin (41) and other inhibitors of

VEGF signaling in the near term, with the prospect of positively impacting treatment of late-stage disease. In the longer term, a new generation of RTKI with dual specificity against VEGF and PDGFRs (42) have similar potential to significantly impact treatment of well-established solid tumors.

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A three dimensional reconstruction of a dormant tumour micrometastasis (150 by 200 μm) of Lewis Lung carcinoma forming a cuff around a small venule in the lung of a mouse. The vessel is in red, apoptotic cells are green and the tumour metastasis is aqua.

Angiogenesis in cancer, vascular, rheumatoid and other disease

Recent discoveries of endogenous negative regulators of angiogenesis, thrombospondin, angiostatin and glioma-derived angiogenesis inhibitory factor, all associated with neovascularized tumours, suggest a new paradigm of tumorigenesis. It is now helpful to think of the switch to the angiogenic phenotype as a net balance of positive and negative regulators of blood vessel growth. The extent to which the negative regulators are decreased during this switch may dictate whether a primary tumour grows rapidly or slowly and whether metastases grow at all.

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Cancer metastases may present at least four common clinical patterns (Table 1): I, A primary tumour such as a colon carcinoma is removed, but within a few months metastases appear; II, metastases are already present when the primary tumour is first detected; III, metastases appear first and the primary tumour remains 'occult'; IV, the primary tumour is removed (or treated by other therapy) and metastases do not appear for several (5–10) years. A fifth but rare pattern is also recognized: metastases disappear after removal of the primary tumour (as seen, for example, with a few cases of renal cell carcinoma).

These patterns of metastatic presentation are well recognized, but their biological basis is poorly understood. Why do some metastases appear in the lung or liver years after removal of the primary tumour? These are called 'dormant' metastases, but what is tumour dormancy? Can this long latency be attributed simply to slow proliferation of the metastatic cells or to the absence of proliferation? If so, why do these metastases, once detected, seem to grow at about the same rate as metastases that appear only months after removal of the primary tumour[69]? Local or regional recurrence of breast cancer in a mastectomy wound has been shown to result from a period of dormancy followed by rapid growth, and not from uninterrupted constant growth[1]. Do metastases follow a similar path? Several reports suggest that this might be the case and offer hypotheses to explain tumour dormancy and escape from dormancy on a hormonal[2], immunological[3] or cell cycle basis[4,5,6]. But, no unifying cellular or molecular mechanism has been put forward. Until recently, endogenous angiogenic control of metastatic growth would be an unlikely candidate, but new experimental evidence lends itself to a hypothesis that says that the majority of the presenting patterns of metastases may be dictated by the intensity of angiogenesis in their vascular bed.

Angiogenesis, positive and negative factors

Several lines of experimental evidence support the angiogenesis hypothesis. Human tumours and spontaneously arising animal tumours are not usually angiogenic at the beginning of their development. *In situ* carcinomas may exist for months or years without neovascularization and as a consequence they remain limited to a small volume of a few cubic millimeters. Some tumour cells then switch to the angiogenic phenotype and recruit new capillary blood vessels that support the growth of both the angiogenic and non-angiogenic cells[7-11]. In breast biopsies it is possible to see *in situ* carcinomas before and after the switch to the angiogenic phenotype[9]. The new capillary sprouts and loops become surrounded by cylinders of tumour cells.

Expansion of tumour mass is made possible not only because of perfusion of blood through the tumour, but also because of the paracrine stimulation of tumour cells by numerous growth factors and matrix proteins that are produced by the new capillary endothelium[12-14]. The switch to the angiogenic phenotype itself depends on a net balance of positive and negative angiogenic factors released by the tumour[15,16,17]. The positive factors include aFGF[18], bFGF[19], VEGF[20], angiogenin[21] and others[22]. They can be exported from tumour cells, mobilized from extracellular matrix or released by macrophages attracted to the tumour.

Although this increased production of positive angiogenic factors, is necessary, it is not sufficient for the angiogenic phenotype. Negative regulators must be decreased. Of several naturally occurring negative regulators of angiogenesis[5,22], thrombospondin was the first for which it was demonstrated that the inhibitor is produced constitutively by normal cells but downregulated during tumorigenesis[15]. Thus, by the time tumour cells have become angiogenic, they are producing only 4–6% of the thrombospondin originally generated by their normal precursor cells. Of great interest is that in human fibroblasts this angiogenesis inhibitor is normally under the control of the *p53* tumour-suppressor gene. Fibroblasts from cancer prone patients with the Li-Fraumeni syndrome have only one copy of *p53* and when this allele is mutated or deleted, thrombospondin production is decreased and angiogenic activity switched on[17]. Despite the fact that individual angiogenic tumour cells may have downregulated production of thrombospondin, a tumour mass of 1 cm^3 containing approximately a billion cells could still generate significant quantities of such an inhibitor. This has been formally demonstrated for a novel angiogenesis inhibitor, angiostatin, that accumulates in the circulation in the presence of a growing primary tumour and disappears when the tumour is removed[23]. Angiostatin is a 38 kD protein with more than 98% homology to an internal fragment of plasminogen. It has an N-terminus at amino acid 98 and an approximate C-terminus at amino acid 440 (ref. 23). It is a specific inhibitor of endothelial cell proliferation and appears in the serum generated only in the presence of the primary tumour. Angiostatin disappears from the circulation five days after removal of the primary tumour (and intense angiogenesis in the metastases is then followed by their rapid growth). While it is not yet clear whether angiostatin is produced directly or indirectly by the primary tumour, the inhibitor profoundly suppresses angiogenesis in remote metastases. A third angiogenesis inhibitor which appears to be downregulated in a human brain tumour and upregulated by restoration of wild-type *p53* tumour-suppressor gene has recently been reported[24].

Switching on angiogenesis

Metastases themselves are dependent on angiogenesis at two stages of the metastatic cascade[5,25,26]. First, typically metastatic cells are not shed from a primary tumour until after it has become neovascularized[65]. Second, upon arrival at their target organ, metastatic cells must again undergo neovascularization if a metastasis is to grow to a clinically detectable size. In the absence of angiogenic activity, metastatic tumour cells may form microscopic perivascular cuffs around the microvessel from which they presumably left the circulation. A detailed analysis of these dormant metastatic colonies in the mouse lung reveals that: they are less than 0.3 mm in diameter; they have a high replication rate, a high death rate by apoptosis, and they are not neovascularized[23,27].

Metastatic patterns in cancer patients

At first diagnosis: Primary tumour	Metastases	Recurrence of metastases
I +	0	months
II +	+	
III 0	+	
IV +	0	years
V +	+	

(Mestastases regress when primary tumour removed: renal carcinoma, rare.)

Table 1. Representation of the most common patterns of presentation of metatsases at the time of first diagnosis of cancer and subsequently at the time of recurrence of metastases (or first appearance of metastases). +, Primary tumour or metastases are detectable; 0 , primary tumour or metastases are not detectable.

This form of dormancy profoundly limits the expansion of a metastasis until angiogenesis occurs (despite the high replication rate of its tumour cells). In one type of mouse tumour (Lewis lung carcinoma), the onset of angiogenesis can be triggered by removal of the primary tumour, which leads to the disappearance of angiostatin from the circulation. In a variant of Lewis lung carcinoma, metastases become angiogenic and grow rapidly whether or not the primary tumour is in place, and angiostatin is not detected in the circulation even in the presence of this tumour type[23]. In yet another mouse tumour (a subclone of B-16 melanoma), metastases may not become angiogenic even 3.5 months after removal of the primary tumour. In this situation, the switch to the angiogenic phenotype appears not to depend on a decrease in endogenous circulating inhibitor(s) associated with the primary tumour, but instead on an intrinsic program in the metastatic cells. Experiments in transgenic mice bearings spontaneous tumours, indicate that not all the cells of a tumour switch to the angiogenic phenotype. Only a subset of cells (as few as 4–10%), become angiogenic[7,8]. Similarly, in human primary tumours, there are microscopic areas of highly intense neovascularization contiguous to areas of lesser neovascularization[9], suggesting a heterogeneity of clones of highly angiogenic cells as well as clones of tumour cells that are weakly angiogenic or not angiogenic. Thus, it is probable that non-angiogenic tumour cells which escape from a primary tumour may remain non-angiogenic in the metastatic site for a prolonged time until they themselves switch to the angiogenic phenotype. The actual mechanisms by which individual tumour cells make this switch are not understood. However, the essential role that angiogenesis plays in the metastatic cascade can be appreciated by examining animal models that have been developed for each of the common presenting patterns of metastases in cancer patients.

The patient whose metastases appear within a few months after removal of the primary tumour (group I, Table 1) is analogous to a

mouse model of Lewis lung carcinoma in which lung metastases remain microscopic as long as the primary tumour is present, but grow rapidly a few days after the primary is removed. In this model, the primary tumour directly inhibits angiogenesis in the bed of the lung metastases. The metastases remain avascular[23] and restricted to a radius of approximately 150 μm. Angiogenesis in the primary tumour is mediated mainly by vascular endothelial growth factor (VEGF), which is presumably present at higher concentrations than local angiostatin. It is also possible that VEGF is retained in the vascular bed of the primary tumour or may be upregulated by hypoxia.[28,29]. The half-life of VEGF in the circulation is approximately three minutes[30]. This rate of clearance would prevent VEGF from accumulating in the plasma. In contrast, the half-life of angiostatin is 2.5 days, and it does accumulate in the serum with increasing size of the primary tumour[23].

The patient whose metastases are already present when the primary tumour is first diagnosed is analogous to a mouse model in which a subclone of Lewis lung carcinoma forms a primary tumour that does not suppress its lung metastases and does not generate detectable levels of angiostatin in the circulation[23].

The patient who presents with metastases in the absence of a detectable primary tumour ('occult primary') is similar to a mouse model in which metastatic cells inhibit the growth of the primary tumour (although it was not determined if the inhibition was mediated by a circulating angiostatic protein[31]). We speculate that if metastases in a patient are shed from a small primary tumour soon after it becomes neovascularized, the tumour may not be large enough to suppress angiogenesis in remote metastases. In mice with angiostatin-generating tumours, the primary tumour had to be at least 0.6–1.0 cm^3 before angiostatin could be detected in the circulation[23]. Furthermore, if the metastases have a slightly faster proliferation rate than the primary tumour, they could produce sufficient quantities of circulating angiogenesis inhibitor and suppress the primary tumour, an example of a secondary tumour suppressing its primary lesion.

The patient whose metastases do not appear until years after removal of the primary tumour is analogous to a mouse model of B16 melanoma, in our laboratory, in which dormant, non-angiogenic lung metastases of less than 0.1–0.2 mm diameter were found as late as 3.5 months after removal of the primary tumour. The mice were healthy[72]. This is equivalent to ten years of human life-span.

Although no animal model has been developed for the rare case of renal cell carcinoma in which removal of the primary tumour is followed by regression of lung metastases, one can speculate that the metastases may have been dependent upon high production of circulating angiogenic factors and possibly other growth factors from the primary tumour[31]. In renal cell carcinomas, high tissue levels of the angiogenic polypeptide basic fibroblast growth factor (bFGF) correlate with high mortality[32]. In fact, in our own study of bFGF in serum and urine, 10% of a group of patients with a wide spectrum of malignancies had abnormally elevated levels of bFGF in their serum and 37% of 950 patients had abnormally elevated levels of bFGF in urine[33].

However, the similarity of these and other animal models to human patterns of metastasis presentation does not prove that angiogenic control of metastatic growth is a central mechanism of dormancy. Nor does it mean that the human patterns are all based on angiogenic mechanisms. These models are described here because they offer a plausible general mechanism to explain the different patterns of metastasis presentation in cancer patients. The detailed experimental evidence is developed elsewhere by Holmgren *et al.*[27]. Further attempts to uncover evidence that supports or rejects the hypothesis may be fruitful. Finally, to the extent that angiogenic processes are operating in human primary tumours and metastases, it may be prudent to include this in thinking about the design of clinical trials of angiogenesis inhibitors.

Clinical implications

From at least eight clinical trials of different angiogenesis inhibitors that are already underway in the U.S., U.K. and elsewhere in Europe, and from extensive pre-clinical studies of angiogenesis inhibitors in animals, certain general principles have emerged[10]. These principles may serve as guidelines for the use of angiogenesis inhibitors in neoplastic or non-neoplastic disease.

1. Antiangiogenic therapy generally has low toxicity. It is directed mainly at proliferating capillary endothelial cells and does not cause bone marrow suppression, gastrointestinal symptoms or hair loss. (Examples are platelet factor 4 and the fumagillin-derivative AGM1470 (TNP-470)).

2. Antiangiogenic therapy appears to have its optimum efficacy if given daily or intermittently over a long time period (months to a year) without a break in therapy. This conclusion is based on experience with interferon-α-2a therapy of life-threatening or sight-threatening haemangiomas in infants where the inhibitor was administered daily for up to a year[70]. Also, patients with different types of solid tumours were treated for up to seven months with an angiogenesis inhibitor, carboxy-amino-triazole[34,35].

3. Drug resistance has not been a significant problem in long-term animal studies[66] or in Phase I/II clinical trials reported so far[36]. Furthermore, proliferating endothelial cells have developed little or no resistance to angiogenesis inhibitors. Antiangiogenic therapy has also been proposed as a potential strategy to avoid drug resistance[68].

4. Quantitative methods developed to monitor efficacy of antiangiogenic therapy are being tested in clinical trials. These include quantitation of angiogenic peptides in serum[37], urine[33] and cerebrospinal fluid[38], and quantitation of neovascularization in histological sections by microvessel count[9,39,40]. In the treatment of life-threatening haemangiomas in infancy, urinary levels of bFGF have proven to be very valuable in adjusting dosage of the drug and in distinguishing haemangiomas from vascular malformations (which do not respond to interferon or to any known drug)[71].

5. A combination of antiangiogenic therapy and cytotoxic therapy can be curative in tumour-bearing animals, for which either agent alone is only inhibitory[41]. Thus, treatment of the endothelial cell compartment of a tumour as well as its tumour cell compartment may need to be considered as potentially more effective than treatment of either cell compartment alone. Other experimental studies show that angiogenesis inhibitors increase uptake of chemotherapeutic drugs in a tumour (Teicher, B. A., personal communication) and also increase flow (by 'unpacking' tumour cells and reducing interstitial pressure). Therefore, angiogenesis inhibitors can be administered together with chemotherapeutic agents, although it is not necessary for the chemotherapy to precede the administration of angiogenesis inhibitors.

Based on this experience, angiogenesis inhibitors may eventually be used to augment conventional therapy. After the completion of chemotherapy, radiation or surgery, antiangiogenic therapy may be continued for years, to prolong dormancy of microscopic metastases or to stabilize residual disease. Many patients with breast cancer are asymptomatic during the ten or more years between the removal of their primary tumour and the appearance of metastases. This suggests that microscopic, dormant metastases may be analogous to infection without disease (e.g., herpes zoster virus and leishmaniasis). It will be important to determine whether antiangiogenic therapy can prolong dormancy.

Non-neoplastic diseases. The lessons from tumour angiogenesis can

be applied to understanding angiogenesis in physiologic conditions and in non-neoplastic disease. Similar angiogenic factors as those seen in tumour angiogenesis are operating in these conditions as in tumour angiogenesis, although their regulation may be different.

Collateral blood vessels. Formation of collateral blood vessels in ischaemic limbs[28] or in ischaemic myocardium[42] may depend upon the hypoxic upregulation of VEGF production, just as occurs in hypoxic areas of tumours[29]. In fact, experimental collateral vessel formation in ischaemic tissue can be accelerated by intravascular administration of VPF/VEGF[28].

Ocular neovascularization. In certain types of abnormal ocular neovascularization there is increased expression of VEGF in the retina itself, and increased levels of VEGF are found in the vitreous[43,44]. Experimental evidence shows that the retinal ischaemia induced by retinal vein occlusion in monkeys results in a rapid rise of VEGF in the vitreous and anterior chamber which parallels the development of retinal neovascularization[43,44]. VEGF may be a major mediator of ocular neovascularization in diabetes[44]. Because of their safety and relatively low toxicity, certain angiogenesis inhibitors developed for anticancer therapy may be used to treat ocular neovascularization.

Infantile haemangiomas Both VEGF and bFGF are overexpressed in infantile haemangiomas during the proliferative phase, as determined by immunohistochemical staining of histological sections[45]. During the involuting phase of these haemangiomas, expression of VEGF decreases to a normal level, followed by a decrease of bFGF[45]. Furthermore, urinary levels of bFGF are abnormally elevated during the proliferative phase of haemangioma but return toward normal during involution of the lesions or after therapeutic intervention[71].

Arthritis. In arthritis, the ingrowth of a vascular pannus may be mediated by excessive production of angiogenic factors from infiltrating macrophages, immune cells or inflammatory cells[46]. In experimental immune arthritis in rats, the angiogenesis inhibitor AGM-1470 has been very effective in preventing neovascularization of the joint. No clinical trials have been initiated at the time of writing.

Psoriasis. The balance of positive and negative regulators of microvessel growth is illustrated in psoriasis. Hypervascular psoriatic lesions overexpress the angiogenic polypeptide interleukin-8 and reveal a decreased expression of the angiogenesis inhibitor thrombospondin[47]. Although the conventional view is that the hypervascularity of psoriasis is due to classical angiogenesis, it is possible that the increased elongation and widening of dermal vessels in psoriasis may be a form of 'non-sprouting' angiogenesis[48].

Duodenal ulcers. In the gastrointestinal tract, several diseases are dominated by abnormal regulation of angiogenesis. Experimental duodenal ulcers in rats[49] are relatively deficient in microvessels[50,51]. Human gastric ulcers show a 23-fold decrease in bFGF content (10 ± 2.4 picograms/mg) compared to normal mucosa[52] which contains high levels of bFGF (234 ± 30 picograms/mg). This is supported by immunohistochemistry in rats[51]. Furthermore, oral administration of bFGF, to rats with duodenal ulcers, induces angiogenesis in the ulcer bed. This can result in as much as a 9-fold increase in microvessel density and accelerates ulcer healing[51]. The bFGF was made acid-resistant by site-specific mutagenesis. These data suggest that healing of peptic ulcers may be angiogenesis-dependent, like chronic wounds. There are further similarities between the angiogenesis of chronic wounds and tumours[53]. These laboratory studies have led to two Phase I clinical trials one[54] in Boston, USA and the other[52] in Nottingham, England, in which patients with duodenal ulcers refractory to conventional therapy are treated with oral bFGF. Early reports from both studies show accelerated healing[52], but as yet there are too few patients to determine efficacy.

Female reproduction. Several disorders of the female reproductive system, such as prolonged bleeding, may be due to dysfunction of endogenous angiogenic stimulators or inhibitors. In fact, it appears that physiologic angiogenesis in the female reproductive tract is mediated by angiogenic stimulators and inhibitors similar to mediators of tumour angiogenesis, but under different regulation[55]. Growth of the ovarian follicle and its corpus luteum may be governed by increased angiogenesis which occurs in the dominant follicle[56-58]. Up to half of the cells in the mature corpus luteum are endothelial cells[57]. Involution and atresia of a follicle are also associated with regression of vascularization and a decrease in DNA synthesis of vascular endothelial cells[59]. Heparin-binding growth factors similar to FGF have been identified in the corpus luteum. These correlative observations clearly do not prove that development of the follicle or its corpus luteum are angiogenesis-dependent. (This would require the type of direct experimental evidence that shows that tumour growth is angiogenesis-dependent[60].) Nevertheless, it would be interesting to know if the dominant follicle, which becomes neovascularized during ovulation, can inhibit the neovascularization of other follicles analogous to the way in which a primary tumour inhibits neovascularization in its metastases.

Developmental angiogenesis. It is possible that certain disorders of development (such as intestinal atresia, vascular malformations and unilateral facial atrophy) may be due to angiogenic abnormalities. The recent discovery that the sedative thalidomide is an angiogenesis inhibitor suggests a possible mechanism to explain why it is such a potent teratogen and why it was the cause of limb defects in babies of mothers who took it during early pregnancy[61]. During embryogenesis, the development of the vascular system itself and the vascularization of organs and tissues appears to follow some of the same rules as tumour angiogenesis, but again, under different regulatory programs. Thus, neovascularization of the renal anlage is a paracrine process mediated in part by bFGF, similar to induction of new vessels by a tumour. New capillary vessels arise from pre-existing vessels (angiogenesis)[62]. However, in the embryo, new vessels also arise by the *de novo* differentiation of early endothelial cells, a process called vasculogenesis[63]. VEGF appears to play a major role in the mediation of developmental vasculogenesis as well as angiogenesis[64]. Postnatal vasculogenesis has never been observed but it would not be entirely surprising if it were discovered in a tumour.

Summary

The identification of a new negative regulator of angiogenesis, angiostatin, has provided a concept of tumour dormancy, such as a tumour population in which proliferation and death of tumour cells has reached a steady state. Such a dormant tumour population is held in check at a microscopic size by the absence of angiogenesis or by its limited response. This new perspective that some endogenous negative regulators of angiogenesis circulate, and that others are normally under the control of the *p53* tumour- suppressor gene, suggests that such inhibitors may participate in the suppression of proliferation in normal microvascular endothelium. They may also regulate physiological angiogenesis and perhaps the angiogenesis that occurs in wound repair. Taken together, these findings provide a new approach to the management of metastatic cancer and also indicate how clinical trials for angiogenesis inhibitors may be optimally designed in the future.

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Fundamental Concepts of the Angiogenic Process

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Abstract: The process of angiogenesis encompasses the growth and regression of capillary blood vessels. Angiogenesis is finely regulated at the molecular and genetic levels, not unlike other physiologic processes such as coagulation, glucose metabolism, and blood pressure. During the development of the field of angiogenesis research over the past three decades, fundamental concepts have been introduced along the way in an attempt where possible, to unify new data from a variety of different laboratories. I have assembled here the major concepts which underlie the angiogenic process as we currently understand it. Many of these are now taken for granted, but this was not always the case, and I have tried to show how they were developed. My goal is to provide a conceptual framework for those basic scientists or clinicians who may enter this rapidly expanding field. Each concept discussed here is accompanied by a few key references as a guide to the pertinent literature.

TUMOR GROWTH IS ANGIOGENESIS-DEPENDENT

The idea that tumor growth is angiogenesis-dependent, and the corollary concept that anti-angiogenic therapy could be employed to treat cancer, was first proposed in 1971 [1]. It was based on previous experiments [2], in which murine tumors implanted into isolated perfused canine thyroid glands remained viable, but failed to expand beyond 1 to 2 millimeters diameter. The tiny tumors grew rapidly when transplanted to mice. The difference was that tumors in mice were highly neovascularized, but the tumors in the isolated organs had no blood vessels. The 1971 paper [1] also discussed recent experiments in which tumors grown in the anterior chamber of the rabbit eye beyond the reach of the vascular bed of the iris, remained less than 1 mm^3 in size, but became neovascularized and grew rapidly, after the tumor was implanted on the vascular bed of the iris. This phenomenon was subsequently reported in more detail [3].

Pharmacologic evidence in support of this concept came in the late 1980s and 1990s from inhibition of a wide variety of tumor types by angiogenesis inhibitors such as TNP-470 [4].

Genetic proof that tumors are angiogenesis-dependent was reported by Arbiser et al, who showed that endothelial cells transformed by the SV40 oncogene, became immortal and formed dormant tumors of microscopic size in immunodeficient mice. However, there was no further tumor growth until after the dormant tumors were subsequently transfected by the *ras* oncogene [5]. Chin and DePinho reported that in large melanomas growing under the control of a doxycyline-inducible *ras* oncogene, that down-regulation of *ras* expression caused massive apoptosis of endothelial cells in the tumor's vascular bed within 12 hours, followed by tumor necrosis a few days later. Necrotic tumors underwent complete regression [6]. This experiment demonstrated that it was necessary for an oncogene to be continuously present to maintain the tumor it had induced, and that the *ras* oncogene mediated stimulation of angiogenic endothelium. Watnick et al, reported that *ras* induction of tumor angiogenesis is mediated by down-regulation of thrombospondin which requires cooperation with *c-myc* [7]. The most compelling genetic proof that tumor growth is angiogenesis-dependent comes from experiments reported by Lyden et al, in which tumors are unable to become angiogenic when implanted into transgenic mice in which one allele of the Id1 gene and two alleles of the Id3 gene have been deleted [8]. Subsequent repletion of these mice by transplantation of bone marrow containing normal progenitor endothelial cells carrying Id +/+ and Id3 +/+ genes, permitted neovascularization of the tumors, followed by rapid tumor growth [9].

TUMOR DORMANCY CAN RESULT FROM BLOCKED ANGIOGENESIS

Dormant tumors can be defined by their inability to expand beyond a microscopic size. Stable tumors may be defined by their inability to expand beyond a macroscopic size. It was long assumed that tumor dormancy could only be explained by cell cycle arrest (i.e., a G_0 state), or by 'immune surveillance.' However, blocked angiogenesis has been reported as an additional mechanism of dormancy [3,10-12]. Tumor dormancy by blocked angiogenesis appears to be a more common phenomenon in human tumors. In fact, it is now possible to isolate non-angiogenic tumor cells from human tumors and implant them in immunodeficient mice. These cells form non-angiogenic, dormant tumors of microscopic

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size (< 0.5 mm). This phenomenon has been called "no take," because such dormant tumors are usually invisible until the skin is opened. Beneath the skin lie tiny white dormant tumors. Histology reveals proliferating tumor cells balanced by apoptotic tumor cells and few if any microvessels [12]. These tumors can remain dormant for months to more than a year. Some tumor types spontaneously switch to the angiogenic phenotype within a few months (equivalent to years in a human), while others never switch during the normal life of the mouse (Nava Almog, unpublished data).

NORMAL CELL GROWTH IS CELL SHAPE DEPENDENT

We first demonstrated in 1978 that DNA synthesis is suppressed by restriction of cell spreading and is permitted by cell spreading [13]. Vascular endothelial cells constrained from elongating or spreading were refractory to mitogens in serum and were subsequently shown to be refractory to specific mitogens such as bFGF or VEGF [14]. DNA synthesis and proliferation were prevented regardless of whether the endothelial cells were prevented from spreading by crowding from neighboring cells, or because endothelial cells were plated on a substratum of reduced adhesivity (e.g., tissue culture wells coated with polyhydroxy ethylmethacrylate [polyHEMA]. This phenomenon provides one explanation for the general non-responsiveness of endothelial cells in the normal vasculature to circulating endothelial mitogens or pro-angiogenic molecules. In fact, the growth of new endothelial vascular sprouts from a pre-existing venule is almost always preceded by vasodilation of microvasculature followed by degradation of the basement membrane. Both events permit endothelial cells to elongate and to spread prior to their formation of new capillary blood vessels stimulated by a tumor [15]. DNA synthesis appears in elongated endothelial cells aligned linearly in the central portion of a growing capillary sprout, but not in foreshortened crowded endothelial cells at the proximal end of the sprout. A fundamental difference between normal cells and neoplastic cells is that neoplastic cells proliferate independently of shape constraints.

TUMOR CELLS PRODUCE SPECIFIC ANGIOGENIC PROTEINS

Until the early 1970s it was widely assumed that tumors did not produce specific angiogenic proteins. The conventional wisdom was that tumor vasculature was an inflammatory reaction to dying or necrotic tumor cells. The first endothelial mitogen, bFGF was isolated from the pituitary by Gospodarowicz in 1974 [16] and was subsequently purified from a tumor by Shing *et al.* [17], and then sequenced by Esch *et al.* [18]. Maciag's lab purified aFGF [19]. Dvorak's lab purified VPF (vascular permeability factor) [20], and Ferrara cloned it as VEGF (vascular endothelial growth factor) [21]. Since then, at least 14 pro-angiogenic proteins have been identified which are produced by tumor cells (TABLE 1) [22] and also [23].

Table 1. Positive regulators of angiogenesis most commonly produced by human tumors.

Most commonly produced by human tumors		
VEGF	45,000	Vascular endothelial growth factor
bFGF	18,000	Basic fibroblast growth factor
aFGF	16,400	Acidic fibroblast growth factor
PDGF	40,000	Platelet-derived growth factor
PD-ECGF	45,000	Platelet-derived endothelial growth factor
IL-8	40,000	Interleukin-8
HGF	92,000	Hepatocyte growth factor
EGF	6,000	Epidermal growth factor
Angiogenin	14,100	
Others		
TNF-alpha	17,000	Tumor necrosis factor alpha
TGF-beta	25,000	Transforming growth factor beta
TGF-alpha	5,500	Transforming growth factor alpha
Proliferin	35,000	
PLGF	25,000	Placental growth factor

One or more of these pro-angiogenic proteins may be over-expressed by tumor cells during the switch to the angiogenic phenotype [24]. Overexpression of pro-angiogenic proteins by tumor cells can be triggered by oncogenes, for example, increased expression of VEGF by *ras* [25], or by *bcl-2* [26] (TABLE 2). The fact that VEGF is the major or sole pro-angiogenic protein expressed by breast cancers in 60% of women at the time of first diagnosis, but that other breast cancers can express up to 6 different pro-angiogenic proteins [27], suggests that it may be prudent in the design of a clinical trial of an "indirect" angiogenesis inhibitor, to stratify patients so that the pro-angiogenic protein(s) produced by their tumor (as determined in a biopsy), is matched by an appropriate inhibitor. Indirect angiogenesis inhibitors (i.e, Avastin an antibody to VEGF), target a tumor cell oncogene, or its product, or the receptor for that product [22] [Fig. **1**]. In contrast, "direct" angiogenesis inhibitors (i.e., endostatin, tumstatin or angiostatin), target angiogenic endothelial cells in the tumor, and generally block the endothelial cell from responding (by increased migration or proliferation) to a wider spectrum of pro-angiogenic proteins (Fig. **2**) (TABLE 3).

Table 2. Impact of oncogenes or potential oncogenes on tumor angiogenesis. Assembled from references 22, 25, and 26.

Oncogene	Implicated pro-angiogenic activity
K-ras, H-ras	VEGF upregulation, TSP-1 downregulation
v-src	VEGF upregulation, TSP-1 downregulation
c-myb	TSP-2 downregulation
N-myc	angiogenic properties in neuroblastoma
c-myc	angiogenic properties in epidermis
HER-2	VEGF upregulation
EGFR	VEGF, bFGF, IL-8 upregulation
PyMT	TSP-1 downregulation
c-fos	VEGF expression
trkB	VEGF downregulation
HPV-16	Secretion of VEGF and IFN-a
v-p3k	VEGF production and angiogenesis
ODC	novel angiogenic factor
PTTG1	VEGF and bFGF upregulation
E2a-Pbx1	Induction of mouse angiogenin-3
bcl-2	VEGF upregulation

Table 3. Some examples of direct and indirect angiogenesis inhibitors. From reference 22.

Direct	Indirect
Angiostatin	Anti-VEGF antibody
Arresten	C225
Canstatin	Herceptin
Endostatin	Interferon-alpha
Thrombospondin	Iressa
TNP-470	NM-3
Tumstatin	PTK787
2-Methoxyestradiol	SU-5416
Vitaxin	SU-6668
	SU-11248

Selection of patients for a clinical trial of a "direct" angiogenesis inhibitor may in the future be based on analysis of the endothelial receptors in the patient's tumor. For example, the endothelial receptor for endostatin has recently been reported to be the integrin alpha5/beta1, while the endothelial receptor for tumstatin is alphav/beta3 [28].

Certain pro-angiogenic proteins also induce an increased level of circulating endothelial cells (or possibly progenitor endothelial cells derived from the bone marrow) which can be decreased toward normal by certain angiogenesis inhibitors [29]. If circulating endothelial cells can be shown to be a reliable surrogate marker for efficacy of an angiogenesis inhibitor, then clinical trials may be improved by a combination of (i) stratification of patients according to the pro-angiogenic protein(s) produced by their tumor; (ii) analysis of integrin receptors on endothelial cells in the vascular bed of the tumor, and (iii) quantification of circulating endothelial cells (by fluorescence activated cell sorting).

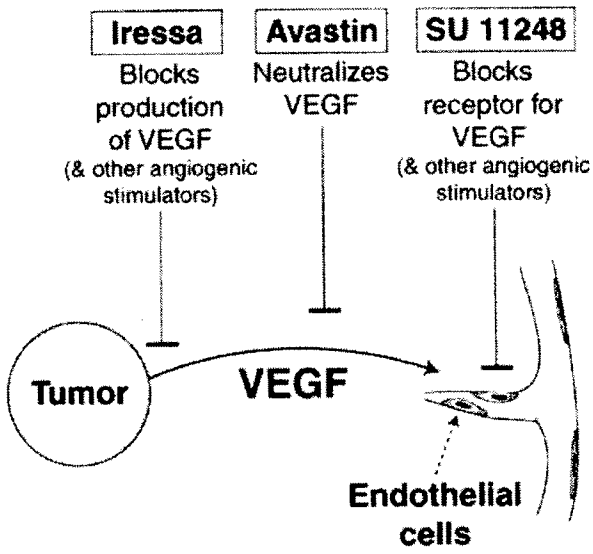

Figure 1. Examples of indirect angiogenesis inhibitors which can block vascular endothelial growth factor (VEGF). Iressa blocks VEGF production from the tumor, as well as blocking other pro-angiogenic proteins. Avastin neutralizes VEGF. SU11248 blocks the receptor for VEGF, as well as the receptors for other pro-angiogenic proteins.

In certain tumors, the angiogenic switch also involves down-regulation of endogenous angiogenesis inhibitors [30], in addition to increased expression of a pro-angiogenic protein. For example, *ras* transfection increases VEGF expression and decreases expression of thrombospondin [22]. In future clinical trials of angiogenesis inhibitors it may be helpful to monitor levels of endogenous angiogenesis inhibitors, but currently this is not as technically feasible as quantifying levels of pro-angiogenic proteins in tumors or in blood or urine [31].

ANGIOSTATIC STEROIDS: ANGIOGENESIS INHIBITORS WHICH ARE ENDOGENOUS

The demonstration that interferon alpha/beta inhibited endothelial cell motility *in vitro* [32] and the subsequent findings that interferon alpha inhibited angiogenesis *in vivo* in mice [33, 34], and in humans [35, 36], and inhibited tumor cell production of bFGF, [37] introduced the idea of the existence of natural

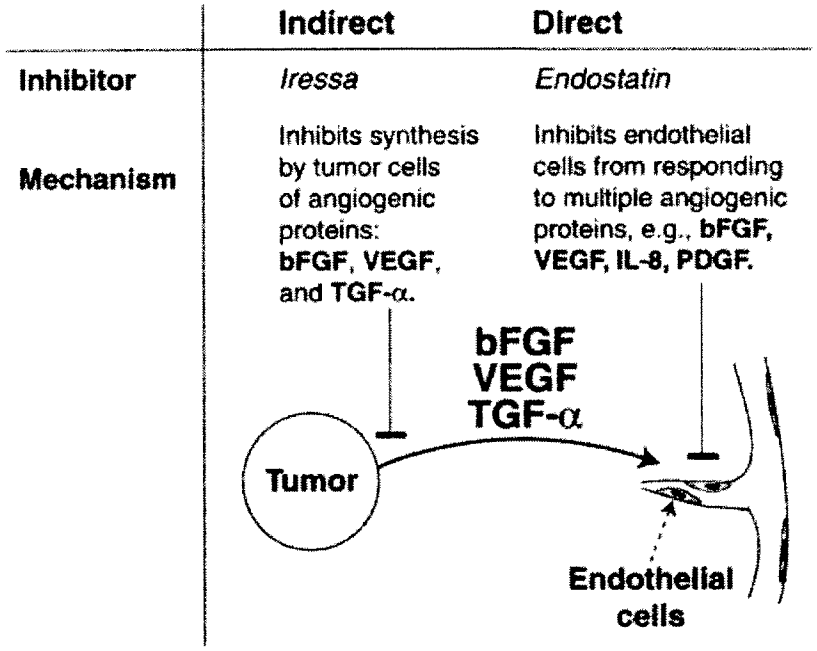

Figure 2. Direct and indirect angiogenesis inhibitors.
Direct angiogenesis inhibitors, such as endostatin, target the microvascular endothelial cells which are recruited to the tumor bed and prevent them from responding to various endothelial mitogens and motogens.
Indirect angiogenesis inhibitors, such as ZD1839 (Iressa), target oncogenes overexpressed by tumor cells, or the products of these oncogenes, or a receptor for these products. Therefore, Iressa targets the tyrosine kinase of the epidermal growth factor receptor and blocks its products bFGF, VEGF and TGF-α.

angiogenesis inhibitors in the body. The subsequent finding that certain corticosteroid metabolites, such as tetrahydrocortisol had antiangiogenic activity without mineralocorticoid or glucocorticoid activity, [38] further supported the concept of naturally occurring angiogenesis inhibitors, and paved the way for the discoveries of angiostatin, endostatin, and other angiogenesis inhibitors which are endogenous. A tetrahydrocortisol analogue is currently in a clinical trial for patients with macular degeneration. The existence of naturally occurring angiogenesis inhibitors which a tumor would have to overcome to induce angiogenesis also formed the basis for the subsequent concept of the 'angiogenic switch' [41, 24]. It is now recognized that at least two endogenous molecular barriers defend against pathological hotspots of angiogenesis: (i) angiogenesis inhibitors in the host such as tetrahydrocortisol, platelet factor 4, angiostatin, and endostatin; and (ii) angiogenesis inhibitors expressed by normal cells, but down-regulated during the switch to the angiogenesis phenotype in tumor cells, such as thrombospondin.

ANGIOGENIC PROTEINS ARE STORED IN EXTRACELLULAR MATRIX

After it was demonstrated that bFGF was stored in the cornea, bound to heparan sulfate in Descemet's membrane [39,40], it became clear that angiogenesis regulatory molecules are present in the body on a 'stand-by' basis. These pro-angiogenic proteins are maintained in a potentially active state and are releasable by specific enzymes or by heparin when angiogenesis is required in physiological processes such as reproduction or repair, and when angiogenesis is induced by pathological processes.

THE ANGIOGENIC SWITCH CONVERTS A NON-ANGIOGENIC MICROSCOPIC DORMANT TUMOR TO A VASCULARIZED GROWING TUMOR

The existence of natural endogenous angiogenesis inhibitors and of angiogenic proteins stored in the extracellular matrix, provided new insights which led to the concept of an angiogenic 'switch.' For a tumor to switch to the angiogenic phenotype, it must overcome two types of natural angiogenesis inhibitors (discussed above): (i) those inhibitors in the host's circulation or extracellular matrix; and/or (ii) those inhibitors in the tumor cell. Rastinejad *et al.* [30], demonstrated that tumor cells did not become angiogenic until they had significantly reduced their own production of thrombospondin. Bouck [41] proposed that the onset of angiogenesis was the result of a shift in the

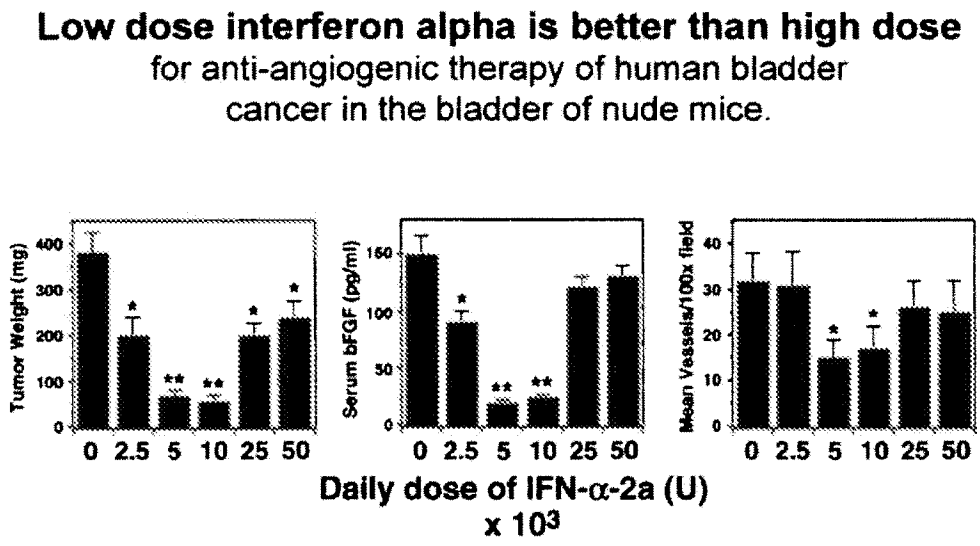

Figure 3. Systemic therapy of bladder tumors in mice with low-dose daily IFN-α-2a. Because of the U-shaped curve which is common to other cytokines, a low daily dose of interferon alpha is more effective than a high dose as an inhibitor of tumor growth, serum bFGF, or angiogenesis (mean vessel density).

balance of positive and negative regulators of angiogenesis. This shift takes place between the pro-angiogenic and anti-angiogenic proteins within the tumor cell itself, and between the tumor cell's angiogenic proteins and the hosts anti-angiogenic proteins [24]. A more detailed outline of the molecular and genetic mediators of the angiogenic switch is beyond the scope of this review, but is discussed in [22] and in [23].

The idea of the angiogenic switch [24] led to a new set of questions:

(i) What happens to a tumor that does not undergo the angiogenic switch? (ii) Can the angiogenic switch be prevented? The first question initiated a new line of research on the mechanism of the stability of the non-angiogenic phenotype in human tumors, i.e., the long time period during which *in situ*, microscopic non-angiogenic tumors remain dormant, (commonly called "no take" when human tumors are transplanted to immunodeficient mice) [12, 42]. Some of these tumors transplanted from human specimens, remain non-angiogenic indefinitely (i.e., more than a year in mice). Others spontaneously switch to the angiogenic phenotype in one to two months (Nava Almog and J. Folkman, unpublished data). Certain dormant human tumors can be rapidly switched to the angiogenic phenotype by transfection with the *ras* oncogene. This results in up-regulation of the tumor cell's production of VEGF and a decrease of its production of thrombospondin. It is now possible to isolate non-angiogenic clones of tumor cells from angiogenic human tumors removed surgically. A provocative finding is that virtually all human tumors examined so far contain significant numbers of non-angiogenic tumor cells. Therefore, the stage is set to experimentally test whether the angiogenic switch can be prevented in the non-angiogenic dormant *in situ* human tumor.

FIRST DEMONSTRATIONS IN MAN THAT HUMAN RECURRENT TUMORS CAN UNDERGO COMPLETE AND DURABLE REGRESSION BY TREATMENT WITH A SINGLE ANGIOGENESIS INHIBITOR

The first use of antiangiogenic therapy in a human was reported in 1989 by Carl White of Denver [35, 43]. (A very low dose of interferon alpha of 3 million units/meter^2/day subcutaneously was chosen during a telephone conversation between White and Folkman). A 12-year-old boy with fatal pulmonary hemangiomatosis had a complete remission and recovered completely after 7 months therapy. Therapy was continued for 7 years. He has been off therapy for 8 years, has graduated college and is working full time in finance. This led to the successful use of low dose daily interferon alpha therapy administered subcutaneously to infants with sight-threatening or life-threatening hemangiomas and hemangioendotheliomas of the heart, airway, and liver [44-48], and to the finding that these lesions over-expressed bFGF [49]. After these lesions underwent complete regression, they did not recur unless treatment time was less than 6 months, in which case treatment was resumed until complete regression.

This experience, coupled with the finding by Singh *et al.* [37] that interferon alpha suppressed expression and secretion of bFGF by human tumors, led us to the successful complete and durable

regression of recurrent high grade giant cell tumors and angioblastomas by treatment with low dose daily subcutaneous interferon alpha [31, 36, 50]. The fact that low dose interferon alpha was effective as an angiogenesis inhibitor in these patients was subsequently substantiated in pre-clinical models in Fidler's lab when they reported that the activity of interferon alpha on tumor growth, serum levels of bFGF and tumor microvessel density, followed a U-shaped curve [51] (see Fig. **3**).

The experience with low dose interferon alpha provided guidelines for subsequent clinical trials of antiangiogenic therapy in other types of cancer:

(i) Frequent dosing without off-therapy intervals is optimally effective.

(ii) Low dose is better than high dose.

(iii) Long-term therapy is necessary, because antiangiogenic therapy is slower than conventional chemotherapy.

(iv) Long-term therapy is feasible because side-effects of antiangiogenic therapy are generally less than with conventional chemotherapy.

(v) A surrogate marker (bFGF in this case), for efficacy of antiangiogenic therapy is valuable for dose adjustment.

(vi) Complete and durable regression was common because the patients were selected to have tumors which produced a single angiogenic protein, bFGF. The expression of this angiogenic protein was inhibited by low dose interferon alpha, frequently administered.

ANEUPLOIDY MAY BE CAUSED BY HORIZONTAL TRANSMISSION OF GENETIC INFORMATION WHEN A TUMOR CELL PHAGOCYTOSES AN APOPTOTIC BODY

One fundamental difference between normal cells and neoplastic cells is that normal cells are genetically stable and maintain a normal karyotype. In contrast, tumor cells are genetically unstable. In the cells of most common human tumors, there is an abnormal karyotype from one cell to another. This genetic instability contributes to the high risk of acquired drug resistance when any therapy is used which attacks the tumor cell *per se*. Antiangiogenic therapy, however, is based on treating a stable genetic target, the activated microvascular endothelial cell in the tumor bed [52].

The chaotic karyotype of cancers cells is characterized by aneuploidy. Recently, Lars Holmgren discovered a novel mechanism of aneuploidy while working as a post-doctoral fellow in the Folkman lab [53]. He continued to study this phenomenon and subsequently demonstrated that while most neoplastic cells which phagocytose an apoptotic body of a neighboring cell, can incorporate DNA and its genetic information by horizontal transfer, apparently only those tumor cells which are p53 -/- can vertically transmit this new genetic material to daughter cells [54]. Cells with functioning p53 appear to be killed when they try to proliferate while carrying abnormal DNA.

PRIMARY TUMORS CAN SUPPRESS GROWTH OF SECONDARY METASTASES BY AN ANGIOGENIC MECHANISM

It is well known among surgeons that removal of certain primary tumors may lead to rapid growth of secondary metastases, reviewed in [55]. A novel mechanism to explain this has been reported [55, 56, 11]. Primary tumors express and secrete high levels of pro-angiogenic proteins, e.g., VEGF. But, these tumors also generate angiogenesis inhibitors by enzymatic cleavage of cryptic fragments from large proteins, (i.e., angiostatin from plasminogen and endostatin from collagen XVIII). Because VEGF has a short half-life in the circulation (minutes) and the angiogenesis inhibitor proteins have a longer half-life in the circulation (hours), the inhibitor accumulates in the plasma in excess of the stimulator and prevents the induction of angiogenesis by micrometases already in place. In the primary tumor however, the angiogenic stimulator (e.g., VEGF) would be in excess of the inhibitor, thus allowing continued growth of the primary tumor while the secondary metastases are suppressed. Surgical removal of the primary tumor causes a decrease in circulating angiogenesis inhibitor with a resultant induction of angiogenesis in pre-existing micrometastases. The same effect can be demonstrated when a primary tumor is regressed by ionizing irradiation [57]. Additional evidence for this concept is provided by experiments in which the inhibitor (angiostatin) is overexpressed by gene transfer in all tumor cells [58].

The elucidation of this phenomenon led to the discovery of the first cryptic angiogenesis inhibitors which are endogenous. It also provided an explanation of the experimental phenomenon of "concomitant tumor resistance," in which tumor cells are inoculated on both flanks of a mouse, but only one tumor grows. When that tumor is surgically removed, the tumor on the opposite flank grows. It also provides a biological explanation for 4 common patterns of presentation of metastases, either by angiogenic regulation of metastatic dormancy or by escape from it [59].

CRYPTIC FRAGMENTS OF MATRIX PROTEINS ARE SPECIFIC INHIBITORS OF ANGIOGENESIS

The discovery of endostatin [56], revealed that a cryptic fragment of collagen XVIII inhibited angiogenesis, whereas the parent protein did not. This suggests a role for matrix proteins in the regulation of angiogenesis [60]. The discovery of

tumstatin in collagen IV by Kalluri's lab [61, 62], further implicated basement membrane proteins as regulators of angiogenesis.

When these proteins are considered together with angiostatin, and antiangiogenic anti-thrombin III [63] a new paradigm is revealed that some proteins (e.g., plasminogen and basement membrane) harbor "unique properties that are cryptic and become exposed only upon proteolytic degradation" [23].

Mice from which tumstatin has been deleted demonstrate the effect of the loss of a cryptic angiogenesis inhibitor. Tumstatin is contained in the alpha 3 chain of collagen IV. When the gene for this chain is knocked out, tumstatin blood levels in mice which are normally in the range of 336 +/- 28 ng/ml decrease to 0 ng/ml and tumors grow 300% to 400% faster, reaching 7 cm^3 in 26 days [64]. However, if tumstatin is replaced at physiological levels (i.e. to achieve normal blood levels), tumors return to the same slower growth rate as in the wild type mice. This fulfills the classic paradigm of a tumor suppressor protein (like p53), except that tumstatin is purely antiangiogenic and has no other known functions). In Kalluri's studies [64] the angiogenesis inhibitor (the ligand) has been knocked out, its receptor has been knocked out, and the enzyme which releases the ligand from its matrix has been knocked out. All models predictability show the same effect of increased angiogenesis, followed by increased tumor growth. Wound healing and pregnancy are not affected. These experiments provide genetic evidence that a normal physiological function of an endogenous angiogenesis inhibitor may be to defend against pathological angiogenesis.

LEUKEMIA IS ANGIOGENIC

The demonstration that human leukemia and other hematological malignancies are angiogenic (in vivo) [65-73], expanded the concept that tumors are angiogenesis-dependent, to the possibility that leukemia and other hematological malignancies may also be angiogenesis-dependent. Further evidence for this hypothesis has recently been published [65] (for review see Folkman and Kalluri Cancer Medicine page 734). A recent study reported that retroviral gene transfer of a vector encoding the direct angiogenesis inhibitors angiostatin and endostatin inhibited bone-marrow angiogenesis and tumor growth in a mouse model of leukemia [74]. Mice inoculated with B-cell, T-cell or myelogenous leukemias and treated with recombinant endostatin have also been observed to live significantly longer and experience fewer toxic side effects than with conventional chemotherapy (Timothy Browder et al, unpublished studies). Taken together, these data suggest that leukemias may be angiogenesis-dependent and may be susceptible to antiangiogenic therapy.

ENDOTHELIAL CELLS APPEAR TO CONTROL SIZE OF NORMAL TISSUE MASS

If microvascular endothelial cells control the growth of virtually all malignancies, we can ask if endothelial cells also control growth of normal tissue mass. Three pieces of experimental evidence indicate that they do. Testosterone induces the up-regulation of VEGF in prostate which leads to angiogenesis in the gland [75]. Proliferating endothelial cells release a variety of mitogens and survival factors which may coordinate prostate hyperplasia with the growth of its endothelial cell population. Furthermore, a natural endogenous angiogenesis inhibitor, pigment epithelium – derived factor (PEDF), regulates the vasculature and mass of the prostate and pancreas [76]. In leptin knockout mice gain of body fat increases continuously and is accompanied by parallel growth of capillary blood vessels. When these mice are treated with an angiogenesis inhibitor (e.g. endostatin or TNP-470), endothelial cells in the adipose tissue undergo apoptosis. The adipose tissue involutes and mice lose weight steadily (but stop losing weight at a physiologically appropriate weight for age [77]). Liver regeneration after hepatectomy in mice has also been reported to be associated with angiogenesis or is angiogenesis-dependent [78-80]. Therefore, it is possible that all tissue mass, whether it is neoplastic or normal, may be regulated by microvascular endothelial cells.

OPTIMUM ANTIANGIOGENIC THERAPY IS ACHIEVED BY CONTINUOUS LEVELS OF ANGIOGENESIS INHIBITOR

A major difference between conventional chemotherapy and antiangiogenic therapy is that chemotherapy has traditionally been administered at maximum tolerated doses with off-therapy intervals of days to weeks, designed to rescue bone marrow and to allow restoration of gastrointestinal epithelium. However, optimum antiangiogenic therapy provides a continuous blood level of the angiogenesis inhibitor. The rationale for this is that tumor-derived pro-angiogenic molecules which continually bathe microvascular endothelial cells, will be opposed by angiogenesis inhibitor molecules. The most compelling of many experiments which support this concept is that continuous administration of endostatin by a micro-osmotic pump in the peritoneal cavity of mice bearing subcutaneous human pancreatic cancer, was 10-fold more effective at inhibiting tumor growth than the same dose given as a bolus injection once per day [81]. Continuous therapy led to tumor regression, bolus once/day therapy did not. Patients receiving endostatin subcutaneously twice daily by a subcutaneous, sustained-release formulation, attain steady state blood levels which are very similar to those attained by continuous intravenous therapy.

CYTOTOXIC CHEMOTHERAPY IS ANGIOGENESIS-DEPENDENT, IN PART

If antiangiogenic therapy is best administered on a schedule that permits continuous exposure of the activated microvascular endothelium in the tumor bed to angiogenesis inhibitor(s), would administration of conventional chemotherapy on a similar schedule improve efficacy of chemotherapy or convert a drug resistant tumor to a drug responsive tumor? Timothy Browder in the Folkman lab answered this question by mouse experiments in which tumors which had failed to respond to "conventional scheduling" of cyclophosphamide (i.e., every other day for 3 days, followed by 21 days off therapy and then the cycle was repeated), regressed when mice were treated on an antiangiogenic schedule of cyclophosphamide administered every 6 days at a lower total dose [82]. Endothelial cell apoptosis in the tumor bed was followed within 4-5 days by apoptosis of tumor cells surrounding each capillary with apoptotic endothelial cells. This report was confirmed and extended by Kerbel's lab [83], using etoposide instead of cyclophosphamide. In a subsequent editorial by Douglas Hanahan [84], this approach was termed "metronomic" chemotherapy. The mechanism of endothelial apoptosis in low dose (metronomic) (anti-angiogenic) chemotherapy remains to be elucidated. Nevertheless, it will be interesting to see if this change in dose and schedule of conventional chemotherapy will by-pass drug resistance because the endothelial cell is the direct target of therapy instead of the cancer cell.

CANCER MAY BE CONVERTED TO A CHRONIC MANAGEABLE DISEASE

As angiogenesis inhibitors become more widely used in anti-cancer therapy, It will be important to determine: (i) Can the harsh side-effects of conventional chemotherapy be reduced? (ii) Can the risk of drug resistance be reduced? (iii) Can cancer eventually be converted to a chronic manageable disease, like heart disease or diabetes? [85]

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Commentary

Molecular Framework for Angiogenesis

A Complex Web of Interactions between Extravasated Plasma Proteins and Endothelial Cell Proteins Induced by Angiogenic Cytokines

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In this issue, the report by Dellian et al[1] describes novel quantitative assays that permit continuous monitoring and characterization of angiogenesis in adult mice. The authors describe the employment of these assays to test an intriguing hypothesis that the physiological properties of newly formed vessels are determined more by vessel origin and microenvironment than by the initial angiogenic stimulus. Their findings support this hypothesis, illustrating that both basic fibroblast growth factor (bFGF) and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) comparably provoked angiogenesis in two different tissues. Moreover, their findings indicate that the rates at which new blood vessels developed in response to each of these cytokines and the physical characteristics of the new vessels were dependent on the location at which the cytokines were implanted. Thus, for example, both bFGF and VPF/VEGF visibly promoted development of new blood vessels from the vascular membrane of the brain within 4 days, whereas new vessels arising from subcutaneous tissue in response to either cytokine were first visible at 10 days. In addition, the new blood vessels formed in response to either bFGF or VPF/VEGF were both hyperpermeable to albumin. Moreover, similar to the rate at which new blood vessels appeared, the level of hyperpermeability displayed by newly formed vessels was dependent on the site at which angiogenesis was provoked but largely independent of the angiogenic stimulus.

Both VPF/VEGF and bFGF are thought to act directly on endothelial cells (ECs) to stimulate angiogenesis,[2] but in contrast to bFGF, VPF/VEGF is also a potent vascular permeability factor that increases microvascular permeability to blood plasma proteins within minutes.[3-6] VPF/VEGF acutely induces microvascular hyperpermeability, with a molar potency 50,000 times greater than that of histamine,[4] through functional activation of vesicular-vacuolar organelles present in the cytoplasm of ECs lining venules and small veins.[7,8] VPF/VEGF has also been implicated in the induction of inter-EC gaps and endothelial fenestrations.[9] Thus, the findings of Dellian et al[1] that VPF/VEGF induced the growth of hyperpermeable vessels are consistent with the known functions of this cytokine. However, because bFGF does not display acute vascular permeability-enhancing activity similar to VPF/VEGF,[6] the observation that microvascular hyperpermeability is also associated with angiogenesis stimulated by bFGF indicates that there may be additional mechanisms distinct from those associated with VPF/VEGF that were responsible for this hyperpermeability. Alternatively, bFGF might have induced VPF/VEGF expression in the experimental systems employed. Previously, VPF/VEGF expression has been demonstrated to be inducible by a variety of cytokines,[10-13] and in particular, bFGF was found to promote induction of VPF/VEGF expression in vascular smooth muscle cells exposed to a threshold hypoxic stimulus.[14] Thus, it remains a possibility that bFGF indirectly was responsible for the hyperpermeability associated with the microvascula-

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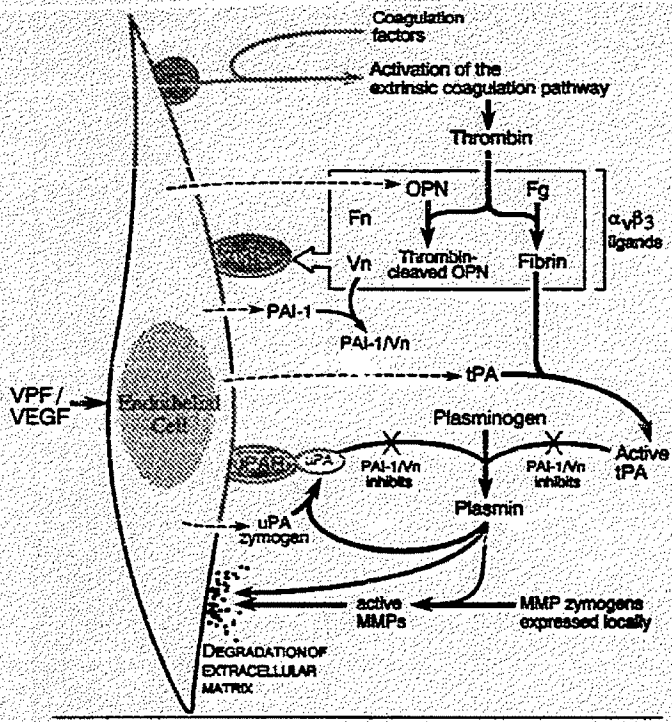
ture by a mechanism involving VPF/VEGF; however, there are also additional possibilities that are not mutually exclusive. For example, dissolution of the vascular basement membrane, EC migration, and EC proliferation are all early characteristic features of angiogenesis,[15] and these processes could contribute to enhancement of vascular permeability by altering vascular structure. bFGF promotes EC proliferation,[2] induces EC expression of proteases,[16–18] and regulates EC expression of cell surface integrins to reduce EC binding to laminin in the basement membrane and to promote EC migration.[19–21] Therefore, at present there appear to be several possible explanations for the association between microvascular hyperpermeability and angiogenesis stimulated by bFGF. Regardless, the findings of Dellian et al[1] indicate that mechanisms by which bFGF promotes angiogenesis result in vascular hyperpermeability, thus drawing a potentially significant parallel with angiogenesis provoked by VPF/VEGF.

Molecular and Cellular Consequences of Microvascular Hyperpermeability for Angiogenesis

As a consequence of microvascular hyperpermeability, blood plasma proteins extravasate.[22] Because microvascular hyperpermeability to plasma proteins is associated with angiogenesis, regardless of whether VPF/VEGF or bFGF is the stimulus,[1] the question arises as to whether there are functional consequences for plasma protein extravasation and, if so, how relevant they are to the development of new blood vessels. Investigations on the induction of gene expression in microvascular ECs by VPF/VEGF and bFGF have yielded several independent findings that offer insights toward the answers to these questions. In particular, the available data collectively suggest the hypothesis that stimulation of angiogenesis by either VPF/VEGF or bFGF involves multiple functionally important interactions between the EC proteins that are induced by these cytokines and plasma proteins that extravasate as a consequence of microvascular hyperpermeability. Figure 1 offers a diagram that summarizes much of the supporting data for this hypothesis. Moreover, this diagram illustrates the potential for complex cellular and enzymatic interactions between proteins from EC and blood plasma compartments involving not only regulation of pericellular proteolysis but also adhesive interactions between the EC surface and components of extracellular matrix. Although Figure 1 specifically relates information that has become available from studies with VPF/VEGF, it appears that many of these relationships are also pertinent to bFGF-driven angiogenesis, as described below.

As indicated on the left side of the diagram, VPF/VEGF stimulation of ECs *in vitro* induces expression of urokinase-type plasminogen activator (uPA),[18] tissue-type plasminogen activator (tPA),[18] plasminogen activator inhibitor-1 (PAI-1),[18] osteopontin (OPN),[23] tissue factor (TF),[24] uPA receptor (uPAR),[25] and the $\alpha_v\beta_3$ integrin.[23] Similarly, bFGF has been reported to induce EC expression of uPA,[17,18] tPA,[18] uPAR,[26] and the $\alpha_v\beta_3$ integrin.[20,21] *In vivo*, VPF/VEGF broadly promotes extravasation of plasma proteins including fibrinogen and coagulation factors,[22] fibronectin, vitronectin (Vn), and plasminogen (A. P. Sergiou and D. R. Senger, unpublished data). Thus, the functional relationships between proteins induced in ECs by VPF/VEGF or bFGF and plasma proteins that extravasate as a consequence of microvascular hyperpermeability can be assigned to three general categories: 1) activation of the extrinsic coagulation pathway and generation of active thrombin from prothrombin, 2) regulation of pericellular proteolysis and degradation of matrix proteins, and 3)adhesive interactions between EC surface integrins and extracellular matrix. However, as illustrated in Figure 1, this categorization is far from absolute and many of the proteins serve functions that relate to more than one category. For example, VPF/VEGF induction of TF and activation of extravasated coagulation factors of the extrinsic coagulation pathway lead to the generation of active thrombin, which converts soluble extravasated fibrinogen to insoluble fibrin,[22] thus modifying the composition of the extracellular matrix. In contrast to soluble fibrinogen, insoluble fibrin markedly enhances tPA activity[27]; consequently, induction of TF by VPF/VEGF could indirectly facilitate the generation of active plasmin from extravasated plasminogen. Plasmin has vast consequences for proteolysis including direct degradation of matrix proteins and activation of locally expressed matrix-degrading metalloproteinases.[28] Interestingly, plasminogen is not only a precursor to plasmin but also the precursor to angiostatin, a M_r 38,000 fragment which inhibits angiogenesis.[29]

Another consequence of activation of extravasated coagulation factors involves cleavage of induced OPN by thrombin. This cleavage enhances both the adhesive and cell-migration-promoting activity of OPN.[23,30] Both intact OPN and its thrombin-cleaved form are ligands for the $\alpha_v\beta_3$ integrin,[30] and this integrin is induced on dermal microvascular ECs by VPF/VEGF[23] and bFGF.[21] The $\alpha_v\beta_3$ integrin also is induced on newly



I) Endothelial cell proteins induced by VPF/VEGF

Secreted	Cell Surface
uPA	TF
tPA	uPAR
PAI-1	
OPN	$\alpha_V\beta_3$

II) Functionally related plasma proteins which extravasate due to microvascular hyperpermeability

Fg
Fn
Vn
Plasminogen
Coagulation factors

Figure 1. *Functional relationships between the proteins induced in endothelial cells by VPF/VEGF and proteins that extravasate from blood plasma as a consequence of microvascular hyperpermeability suggest the hypothesis that multiple and complex interactions between these two protein populations are fundamental to the mechanism by which this cytokine promotes angiogenesis. Although this diagram summarizes data from investigations with VPF/VEGF, most of these relationships apply to bFGF as well (see text). Fg, fibrinogen; Fn, fibronectin; MMP, matrix metalloproteinase. uPAR is also a receptor of Vn (not shown). PAI-1/Vn is a stable complex that inhibits both uPA and tPA. In addition to inhibiting plasminogen activation in the matrix, this complex also likely inhibits pericellular activation of plasminogen through association with Vn receptors at the cell surface.*

formed blood vessels of wound granulation tissue,[31] and antibodies to $\alpha_v\beta_3$ have been shown to block angiogenesis.[32,33] In addition to OPN, the three major adhesive proteins present in blood plasma, fibrin, fibronectin, and Vn, are ligands for the $\alpha_v\beta_3$ integrin, and as noted above, these three proteins extravasate as a consequence of VPF/VEGF-induced microvascular hyperpermeability. Thus, OPN, fibrin, fibronectin, and Vn all likely participate in the regulation of EC adhesion and migration during angiogenesis and ultimately in the development of vascular form.[34] Moreover, the respective contributions of these four ligands to the regulation of these processes are all dependent on a combination of cytokine induction of EC gene expression and microvascular hyperpermeability.

In contrast to fibrin, which indirectly promotes plasminogen activation by enhancing tPA activity,[27] Vn antagonizes plasminogen activation through binding and stabilization of PAI-1.[35,36] PAI-1 inhibits both tPA and uPA,[37] and it is likely that Vn/PAI-1 complexes bound to vitronectin receptors serve to inhibit plasminogen activation at the cell surface.[38] Conversely, vascular uPA and its receptor (uPAR), which, as indicated above, are both induced by VPF/VEGF and bFGF, serve to promote pericellular activation of plasminogen. Although expressed as a zymogen, uPA is readily converted to its active form by plasmin,[39] and therefore the activation of uPA may be indirectly dependent on the extravasation of plasminogen. Although not illustrated in Figure 1, plasma Vn also has been reported to bind to uPAR.[40] Thus, extravasation of plasma Vn may prove to inhibit proteolysis at the EC surface not only through binding and stabilization of PAI-1 but also through binding to uPAR. Vn may also regulate EC adhesion directly through such interactions.

It is likely that interactions illustrated in Figure 1 are relevant to a variety of important pathologies as well as wound healing. Vascular hyperpermeability to macromolecules is a highly characteristic feature of tumors[7,41–47] as well as healing wounds, delayed hypersensitivity reactions, proliferative retinopathies, psoriatic lesions, and rheumatoid arthritis (reviewed in Ref. 7). Moreover, VPF/VEGF has been implicated as pivotally important for promoting the angiogenesis that occurs in tumors,[44,47–55] wounds,[56] delayed hypersensitivity reactions,[57] proliferative retinopathies,[58–62] psoriasis,[10] and rheumatoid arthritis.[63,64] Also, bFGF has been implicated in tumor angiogenesis (reviewed in Refs. 65–67). Recently, and consistent with VPF/VEGF expression in breast carcinomas,[68] TF has been shown to be induced in ECs associated with malignant breast disease.[69,70]

Although it appears that the vast majority of pathologies in which angiogenesis is prominent involve microvascular hyperpermeability as well as VPF/VEGF and/or bFGF, some brain tumors do not exhibit vascular hyperpermeability.[71] However, the absence of microvascular hyperpermeability during tumor angiogenesis in the brain may reflect a highly specific mechanism related to maintenance of the normal blood-brain barrier. Interestingly, relatively high levels of Vn mRNA have been reported to be associated with brain capillaries but not with the vasculature of peripheral organs.[72] Circulating plasma Vn is synthesized primarily by the liver,[73] and presumably the availability of this protein to peripheral tissues is regulated at the level of vascular permeability. However, in the brain, such extravasation is restricted by the blood-brain barrier. Thus, it is tempting to speculate that expression of Vn by brain capillaries is but one example of protein expression by the brain vasculature that is designed to compensate for anatomic restriction against plasma protein extravasation.

Finally, because investigations on the regulation of EC gene expression by VPF/VEGF and bFGF are not complete, it seems highly probable that the functional relationships illustrated in Figure 1 represent only a fraction of the total complexity of such interactions. Thus, it is also likely that more relationships between EC proteins induced by these cytokines and plasma proteins that extravasate will be identified. Moreover, given the potential for considerable complexity and functional overlap, some of these interactions may prove essential for angiogenesis whereas others may not be required. Examples of the latter may include interactions involving either Vn or PAI-1 because homozygous null mice deficient for expression of these proteins develop with an apparently normal vasculature.[74,75] However, it remains a possibility that angiogenesis associated with wound healing or various pathologies may be affected in these Vn- or PAI-1-deficient animals, particularly because the composition of extracellular matrix associated with vascular development in the embryo is very probably distinct from the matrix present in, for example, wounds and tumors.[76,77] Lastly, although this discussion has focused on VPF/VEGF and bFGF, other cytokines such as transforming growth factor-β have also been implicated in the regulation of EC gene expression[78–80] and in angiogenesis.[2] Therefore, in addition to VPF/VEGF and bFGF, other cytokines may also prove to regulate interactions between ECs and extravasated plasma proteins during angiogenesis, and some may prove to regulate such interactions uniquely with distinctly different consequences.

In summary, the report in this issue by Dellian et al[1] presents data illustrating that both VPF/VEGF and bFGF comparably provoked angiogenesis and that the rates at which new blood vessels developed, the physical characteristics of the new vessels, and the levels of hyperpermeability were all largely dependent on the tissue in which angiogenesis was induced but independent of the stimulus. Although bFGF does not display acute vascular permeability-enhancing activity similar to VPF/VEGF,[6] Dellian et al[1] found that the vessels that developed in response to bFGF were hyperpermeable to plasma albumin, raising interesting questions about the basis for this hyperpermeability. In addition, these findings identify an important analogy with angiogenesis stimulated by VPF/VEGF and raise the possibility that extravasated plasma proteins function in bFGF-driven as well as VPF/VEGF-driven angiogenesis. Collectively, the functional relationships between extravasated plasma proteins and the proteins that are induced in ECs by VPF/VEGF or bFGF predict significant involvement of extravasated plasma proteins in neovascularization. In particular, the available data suggest the hypothesis that stimulation of angiogenesis by either VPF/VEGF or bFGF involves multiple functionally important interactions between the EC proteins that are induced by these cytokines and the plasma proteins that extravasate as a consequence of microvascular hyperpermeability. More specifically, this hypothesis predicts that such interactions regulate pericellular proteolysis together with EC adhesion to extracellular matrix.

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Exhibit 2

5.5. Statistics

Unless otherwise noted, the ANOVA or Student t-Test was used for statistical analysis.

6. RESULTS AND DISCUSSION

6.1. Overview of Single Agent Activity and Anti-tumor Efficacy in Subcutaneous Xenograft and Syngeneic Tumor Models

AG-013736 was tested for its single agent anti-tumor efficacy in sc or orthotopically implanted tumor models, including: human colon carcinoma models MV522, HT29, and HCT-116-GFP); human breast carcinoma model MDA-MB-435 HAL-Luc; human SCLC model NCI-H526; murine Lewis lung carcinoma (LLC); human melanoma models A375, M24met, and A2058; human renal carcinoma model SN12C-GFP; human glioblastoma model U87MG; rat glioma model C6; and human non-Hodgkins lymphoma model Namalwa. Table 1 summarizes experimental design, parameters, RTK (receptor tyrosine kinase) expression and efficacy of AG-013736 in these models from a set of representative studies. AG-013736 consistently and significantly demonstrated anti-tumor efficacy in these preclinical tumor models. Importantly, AG-013736 treatment produced significant tumor growth inhibition (TGI) regardless of RTK expression status, suggesting that in the RTK negative tumor models, the anti-tumor efficacy was a consequence of the in vivo anti-angiogenic activity of AG-013736.

6.1.1. Single Agent Anti-tumor Efficacy in Human Colon Carcinoma Models

Studies in the MV522 colon carcinoma model: The MV522 human colon carcinoma model has been used as the primary in vivo model for evaluating the anti-angiogenic and anti-tumor activity of VEGFR inhibitors because: a) in-house data showed that the MV522 line does not express endogenous active VEGFRs, PDGFR-β, or KIT. Therefore, anti-angiogenesis of an agent could be evaluated without the complication of inhibitory signals from both the vasculature and the tumor cells; and b) MV522 xenograft growth has been a reliable in vivo model and experimental results were highly reproducible.

Initial efficacy studies in the MV522 model with AG-013736 demonstrated significant MV522 tumor growth inhibition (76.5%) when dosed at 25 mg/kg, IP, BID (DRS-150). Subsequent studies with this model revealed that AG-013736 induces dose-dependent tumor growth inhibition of 41%, 57%, and 91% when dosed at 5, 10, and 25 mg/kg, IP, BID, respectively (Study DRS-154). Following confirmation of AG-013736 oral bioavailability, a dose escalation study (DDH-119) using AG-013736 formulated in 30:70 of PEG:H_2O was carried out in the MV522 model. AG-013736 treatment via the PO, BID regimen demonstrated dose-dependent tumor growth inhibition (Figure 1A). This result was reproduced in another study, where AG-013736 was formulated in CMC/H_2O and dosed PO, BID at 1, 3, 10, 30, 100, 150, and 200 mg/kg (DDH-MG-371; Table 1). Significant anti-tumor efficacy was observed when AG-013736 was formulated as a solution in a 30:70 of PEG300:H_2O·HCl vehicle (pH 2~3) and delivered via continuous infusion at 1 μL/h using osmotic mini-pumps (DDH-KA-302).

Table 1. Single Agent Anti-tumor Efficacy of AG-013736 in Mice

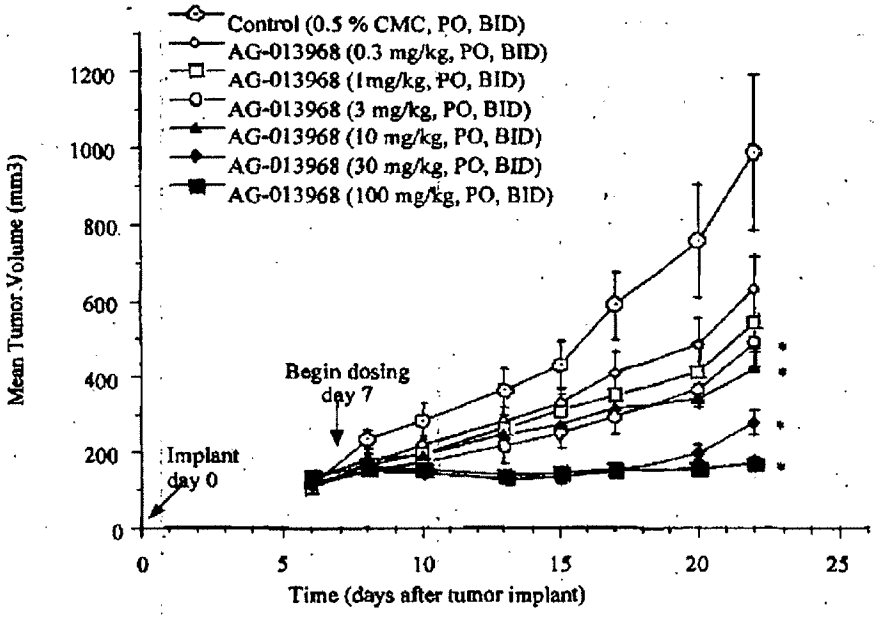
Disease type	Model (Phospho-RTK Expression)*	Description	Dose	Regimen	Size at start of Rx (mm3)	Rx period (Days)	TGI	Study Name
Colon cancer	MV522 (None)	Dose dependent efficacy using AG-013968, the HCl salt form of AG-013736	0.3 mg/kg	PO, BID	160 - 180	16	40	DDH-119
			1 mg/kg				50	
			3 mg/kg				57	
			10 mg/kg				66	
			30 mg/kg				81	
			100 mg/kg				96	
		Dose dependent TGI using AG-013736	1 mg/kg	PO, BID	170	16	18.3	DDH-MG-371
			3 mg/kg				15.2	
			10 mg/kg				63.9	
			30 mg/kg				61.1	
			100 mg/kg				80.3	
			150 mg/kg				94.6	
			200 mg/kg				96.3	
		TGI using Alzet pump delivery of AG 013736	1 mg/mL	continuous infusion	100	14	20	DDH-KA-302
			3 mg/mL				31	
			10 mg/mL				79	
			20 mg/mL				94	
			30 mg/mL				85	
	HT29 (ND)	Dose Response TGI	10 mg/kg	PO, BID	110	16	51	HT29-c143
			30 mg/kg				76	
			100 mg/kg				99	
			150 mg/kg				99	
	HCT-116-GFP (ND)	TGI in orthotopically implanted HCT-116 GFP model	30 mg/kg	PO, BID	One day after implant	10	unevaluatable	HCT-116 GFP Study #3
			30 mg/kg			17	87	
			30 mg/kg			28	80	
Breast cancer	MDA-MB-435 Hal-Luc (None)	Anti-tumor efficacy via bioluminescent signal and conventional caliper measurements	60 mg/kg	PO, BID	90	28	50	DDH-MG-446
			10 mg/kg	PO, BID	130	15	dose dependent reduction in bioluminescent signal	DDH-MG-355
			100 mg/kg					
Lung	LLC (PDGFR-b)	Repeat study on dose dependent efficacy in LLC using AG-013968, the HCl salt form of AG-013736	1 mg/kg	PO, BID	< 50	18	33	DDH-123
			3 mg/kg				49	
			10 mg/kg				75	
			30 mg/kg				68	
			100 mg/kg				69	
			300 mg/kg				not tolerated	
		TGI and anti-metastasis activity assessment	100 mg/kg	PO, BID (trocar implanted)	prophylactic dose	38	63	LLC-DCH-201
			100 mg/kg	PO, BID (tail vein implanted)	--	survival	~ 11 days median survival	
	SCLC (KIT)	efficacy in human SCLC expressing KIT - dose response	30 mg/kg	PO, BID	180	13	47	DDH-MG-293
			100 mg/kg				61	

Table 1 Single Agent Anti-tumor Efficacy of AG-013736 in Mice (continued)

Disease type	Model (Phospho-RTK Expression)*	Description	Dose	Regimen	Size at start of Rx (mm3)	Rx period (Days)	TGI	Study Name
Melanoma	A375 (VEGFR-2, PDGFR-b)	Efficacy in established A375 melanoma	10 mg/kg	PO, BID	336	12	38	DDH-MG-268
			30 mg/kg				59	
	M24met (None)	dose response TGI in sc-implanted M24met	3 mg/kg	PO, BID	120 - 160	13	60	DDH-163
			10 mg/kg				49	
			30 mg/kg				73	
		Anti-metastasis activity in M24met with either early or late start treatments	50 mg/kg	PO, BID	primary tumor resection when ave size ~ 400 mm3	23 days, Early dose (-7 day before surgery)	Sig. reduction in both l.n and lung mets in both groups. Early dosing group had greater effect.	DDH-158B
			50 mg/kg			23 days, Late dose (-1 day before surgery)		
	A2058 (ND)	Dose depended TGI in activating mutation bRaf melanoma	3 mg/kg	PO, BID	150	15	36	DDH-MY-424
			10 mg/kg				57	
			30 mg/kg				65	
			100 mg/kg				83	
Renal cancer	SN12C-GFP (ND)	TGI in orthotopically implanted SN12C GFP human renal carcinoma model	10 mg/kg	PO, BID	Two days after implant	41	54	SN12C-GFP Renal cell carcinoma
			10 mg/kg			72	61	
			30 mg/kg			41	56	
			30 mg/kg			72	74	
			100 mg/kg			41	63	
			100 mg/kg			72	70	
Glioma	C6 (PDGFRs)	Dose dependent efficacy in C6 glioma	10 mg/kg	PO, BID	70 - 80	15	37	DDH-GW-247
			30 mg/kg				47	
			100 mg/kg				64	
	U87MG (PDGFRs)	Dose dependent efficacy in U87 glioma	10 mg/kg	PO, BID	46 - 72	17	44	DDH-GW-273
			30 mg/kg				69	
			60 mg/kg				74	
		Efficacy comparison between single agent AG-013736 and selective VEGFR inhibitor + selective PDGFR inhibitor	100 mg/kg (AG-013736)	PO, BID	50 - 60	13	67	DDH-AA-304
			30 mg/kg VEGFR-selective Inh	PO, BID			51	
			100 mg/kg VEGFR-selective Inh	PO, BID			49	
			20 mg/kg PDGFR selective Inh	PO, BID			22	
			VEGFR (30 mg/kg) + PDGFR	PO, BID			53	
			VEGFR (100 mg/kg) + PDGFR	PO, BID			64	
HNL	Namalwa (ND)	Efficacy in human lymphoma model Namalwa (time course)	50mg/kg	PO< BID		14	Significant reduction in ip tumor burden as indicated by reduction in body weights.	DDH-RF-240
			50mg/kg			22		
			50mg/kg			23		

Figure 1. TGI by AG-013736 in Human Colon Xenograft Tumor Models

A. Dose-dependent TGI in MV522 by AG-013736 via PO, BID



B. Dose-dependent TGI in MV522 by AG-013736 via Mini-Pump Dosing

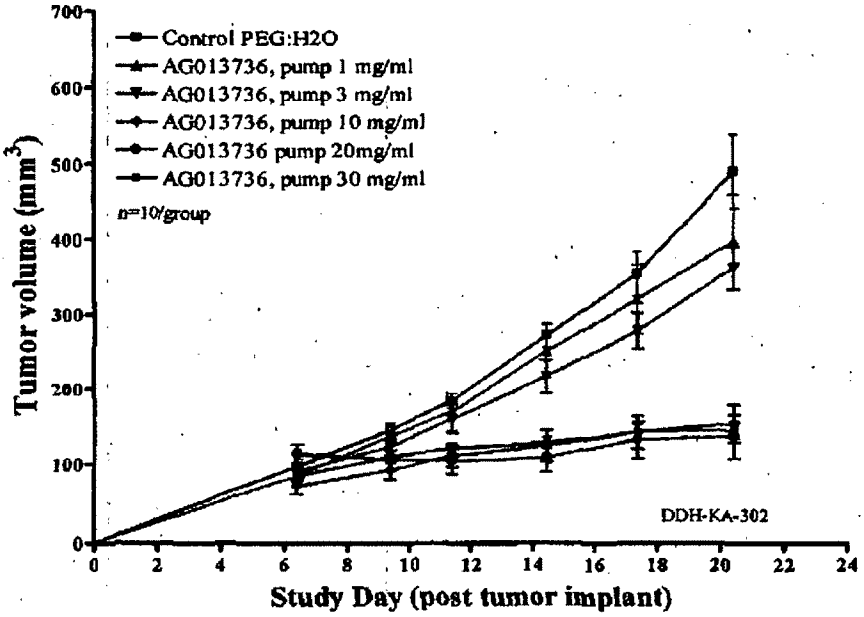
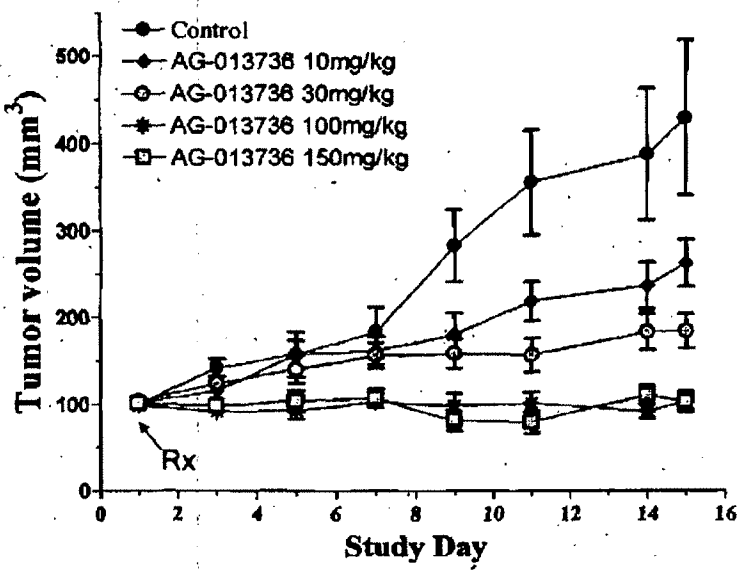
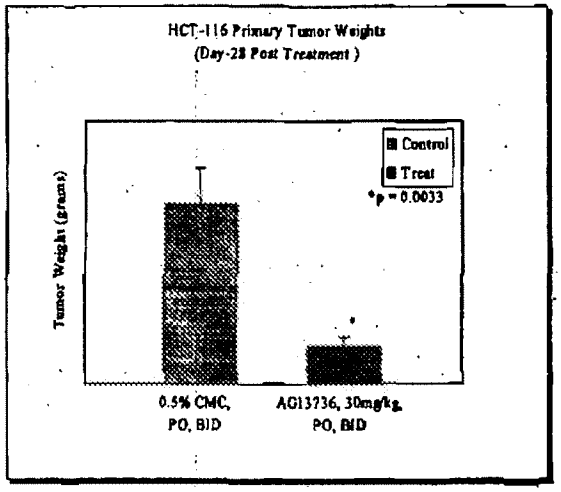


Figure 1. TGI by AG-013736 in Human Colon Xenograft Tumor Models (continued)

C. Dose-Dependent Inhibition in HT29 Colon Carcinoma Model



D. AG-013736 Significantly Inhibited Growth of HCT-116 GFP Tumors Implanted Orthotopically in Colon of Mice



n/group	Control		Treated	
	An#	wt(g)	An#	wt(g)
8	33	0.45	41	0.07
	34	0.37	42	0.11
	35	1.55	43	0.08
	36	1.17	44	0.31
	37	1.06	45	0.43
	38	1.11	46	0.17
	39	0.62	47	0.04
	40	0.29	48	0.14
Mean		0.8275		0.1688
SD		0.4562		0.1345
SEM		0.1613		0.0476
Treat			0.0033	

Significant anti-tumor efficacy of AG-013736 in several human colon carcinoma xenograft models in mice. A. Dose-dependent TGI in the MV522 model when AG-013968 (the HCl salt form of AG-013736) was dosed with the PO, BID regimen; B. similar dose-dependent TGI in the MV522 model when AG-013736 was dosed via continuous infusion using the Alzet osmotic mini-pumps; C. Dose-dependent TGI by AG-013736 (PO, BID) in the HT-29 model; and D. Inhibition of orthotopically-implanted HCT-116 GFP tumor growth in mice by AG-013736 (PO, BID at 30 mg/kg). Left, Change in average tumor weight at the termination of the study; Right, individual tumor weight in control and AG-013736 treated groups at the termination of the study (28 days of treatment, P = 0.0033).

Studies in the HT29 colon carcinoma model: AG-013736 was evaluated in another human colon carcinoma model, HT29. Treatment with AG-013736 at 10, 30, 100, and 150 mg/kg, PO, BID produced a dose-dependent TGI, ranging from 51% (10 mg/kg) to a nearly complete suppression of the growth of the tumor (100 mg/kg). AG-013736 at 100 mg/kg yielded maximum efficacy, i.e., no further increases in TGI were observed at the 150 mg/kg dose level (Figure 1B). The result of this study was consistent with an earlier pilot study HT29-e129. Details of the studies can be found in the study reports (HT29e-129 and 143) by Peidmont Research Center.[10,11]

Anti-tumor activity in the orthotopic model of HCT-116-GFP: AG-013736 was evaluated for its anti-tumor efficacy in an orthotopically-implanted green fluorescence labeled human colon carcinoma model, HCT-116-GFP. Several pieces of HCT-116-GFP tumors were sutured onto the colon of a mouse and AG-013736 was administered the next day at 30 mg/kg, PO, BID. Tumor growth was monitored via non-invasive green fluorescent imaging throughout the treatment period. Animals were taken off the study after 10, 17, or 28 days for open-body evaluation of local tumor growth and metastasis. AG-013736 treatment produced marked primary tumor growth inhibition in comparison to the control group, as demonstrated by statistically significant reduction in primary tumor mass at the termination (Figure 1C; see associated table). The total number of metastasis after 28 days of treatment, including local metastasis in the mesentery and distant metastasis in the lymph nodes and lung, appeared to be decreased from 18 total incidences in the control group to 8 incidences in the AG-013736 treated group. The total green fluorescent signal intensity was not changed between the vehicle control and the AG-013736 treated groups. Overall, this study showed that AG-013736 was effective in delaying primary tumor growth in the colon and effective in inhibiting the total number of metastasis incidence in mice, but was less effective in reducing the severity of local invasion and distant metastasis.

6.1.2. Single Agent Anti-tumor Efficacy in Human Breast Carcinoma Model, MDA-MB-435 HAL-Luc

In two separate studies, AG-013736 was evaluated for its ability to inhibit the growth of human breast carcinoma tumor MDA-MB-435 HAL-Luc (DDH-MG-355 and DDH-MG-446). The cells were engineered to express the luciferase protein once uptake of leuciferin occurred by the proliferating cells. Tumor growth in SCID mice was monitored by measuring the photon emission from viable tumor tissue established either via sc implant or orthotopic implant in the mammary fat pad of the mice. The size of the tumor was also measured using the conventional electronic caliper.

In both studies, AG-013736 significantly reduced tumor growth compared to the control groups, as indicated by the reduction in both the bioluminescent signal and the tumor size. Figure 2A shows a representative set of luciferase bioluminescent images of mice in the vehicle control and AG-013736 treated groups, where the tumors are significantly brighter in the control group than those in AG-013736 treated groups (Study DDH-MG-446). Figure 2B shows the result from caliper measurements. The caliper measurement showed growth delay under AG-013736 treatment whereas the bioluminescence measurements showed growth stasis. The nature of bioluminescent imaging allows for detection of only viable

luciferase-expressing tumor cells within the mass, which has the effect of increasing sensitivity because necrotic sections in the tumor do not contribute to the measured signal. This is in contrast to traditional tumor measurements. Although caliper measurements provide for higher throughput, such measurements cannot discriminate between viable and necrotic parts of the tumor and therefore are at risk of underestimating the anti-tumor effect of treatment. Hence, the bioluminescence measurements in this study more accurately reported anti-tumor efficacy from AG-013736 treatment (Figure 2C). Immunohistology (IHC) analysis showed that AG-013736 treatment significantly reduced microvessel counts in the treated tumors as compared to the control tumors (Figure 2D). Furthermore, the signal of the apoptosis marker, cleaved Caspase-3, was enhanced in the AG-013736 treated tumors (Figure 2E). Consistent with this increased cell death signal, the signal of the cellular proliferation marker, Ki67, was decreased in the treated tumors (Figure 2F).

Figure 2. Tumor Growth Inhibition by AG-013736 in Human Breast Carcinoma Model, MDA-MB-435 Hal-Luc

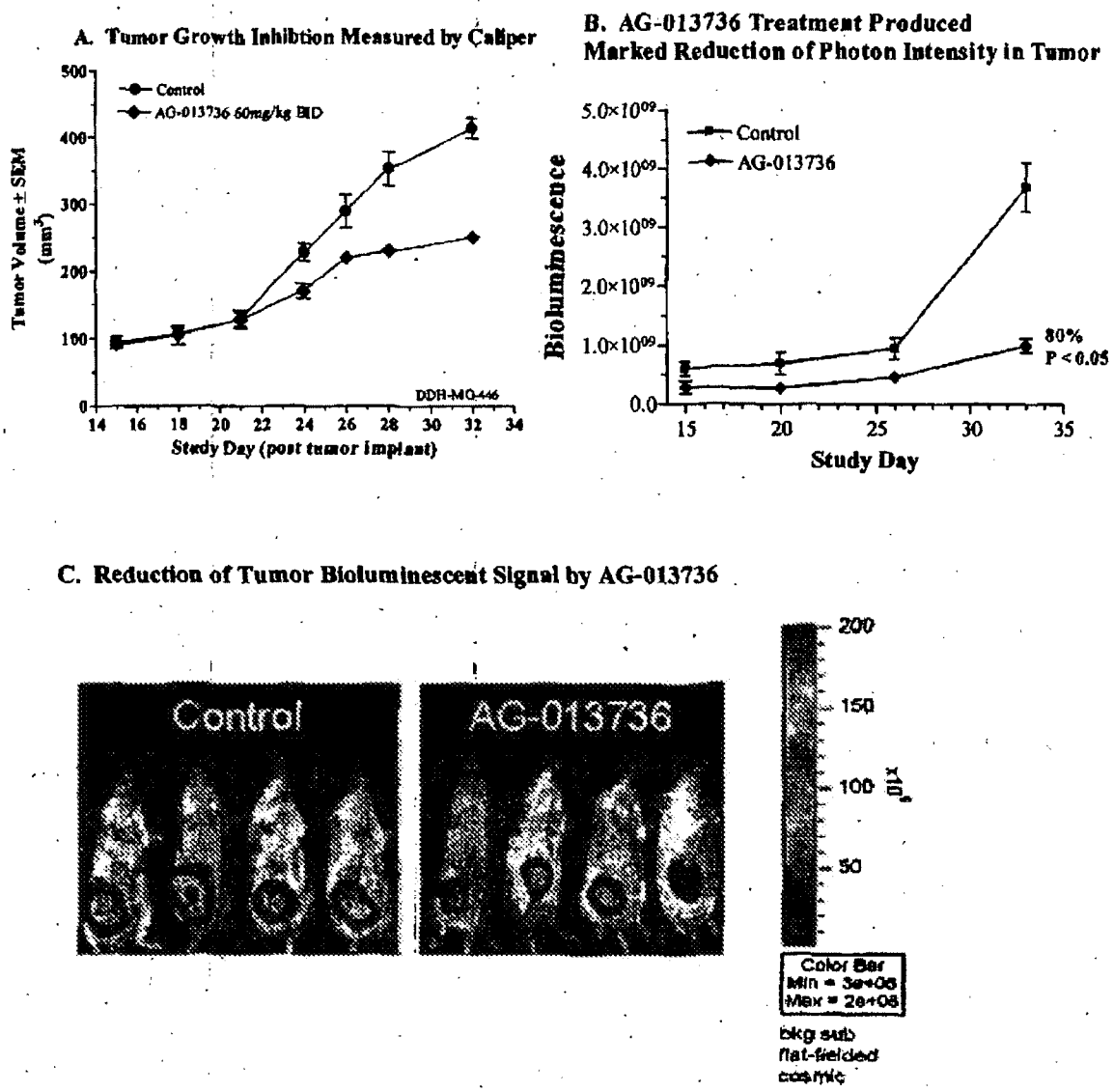
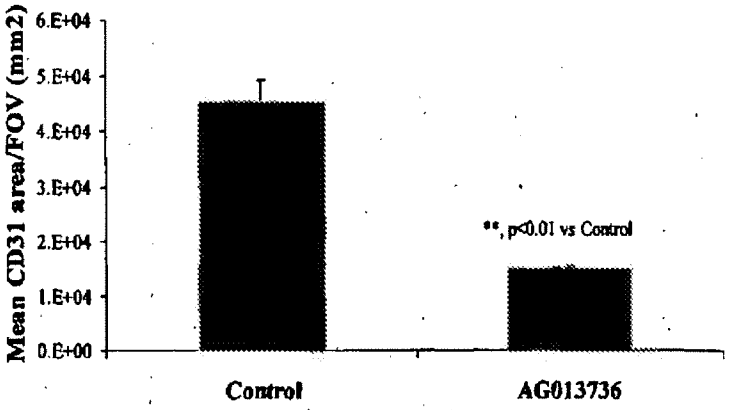
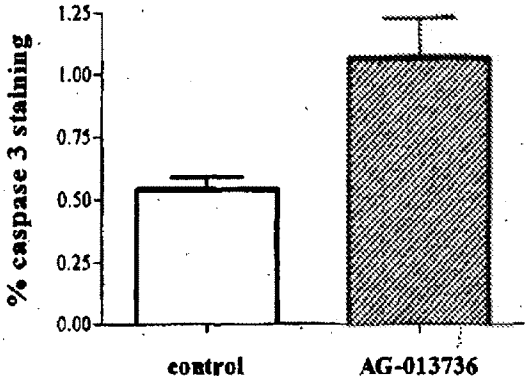


Figure 2. Tumor Growth Inhibition by AG-013736 in Human Breast Carcinoma Model, MDA-MB-435 Hal-Luc (continued)

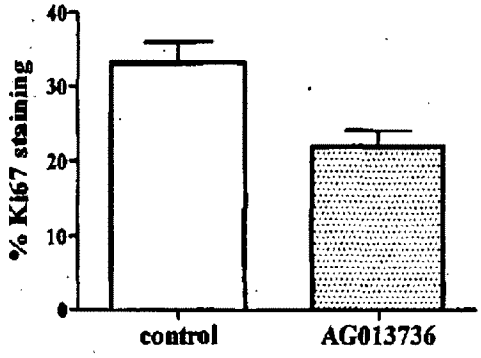
D. Reduction of CD-31 (Angiogenesis) by AG- 013736



E. Caspase3 Induction by AG-013736



F. Reduction of Ki67 by AG-013736



AG-013736 significantly decreased the growth of human breast cancer MDA-MB-435 Hal-Luc in mice. A. A 50% TGI measured by caliper was observed for AG-013736 (60 mg/kg, PO, BID). Dosing started on Day 15 when the average tumor size was about 100 mm^3. B. An 80% TGI anti-tumor efficacy determined by tumor bioluminescence was observed. The initial bioluminescent signal of the control and the treated groups was 6×10^8 photons and 3×10^8 photons, respectively (Day 15). The initial bioluminescent signal for each group was subtracted for TGI calculation. C. Representative image of tumor bioluminescence from the control (left) and the treated group (right) on Day 33 (end of dosing). Tumor tissues were collected and processed for IHC assessment of (D.) reduction in angiogenesis measured by CD-31staining ($P<0.01$ compared to control), (E.) tumor apoptosis as measured by induction of Caspase-3 signal ($P<0.0$ compared to control), and (F.) decreased viable cells in the tumor as measured by Ki67 staining ($P=0.007$ compared to control). Quantitation of IHC signals was acquired using the Automated Cellular Imaging System® (ACIS) by Chromavision (San Juan Capistrano, CA).

6.1.3. Single Agent Anti-tumor Efficacy in Human Lung Carcinoma

AG-013736 was tested for its in vivo activity against murine Lewis lung carcinoma (LLC) and human small cell lung carcinoma (NCI-H526) models in mice. Both models are considered aggressive preclinical tumor models.

Studies in the LLC model: In addition to its aggressive growth nature, the LLC model is a murine carcinoma and the resulting syngeneic model allowed us to assess anti-tumor efficacy of AG-013736 in immuno-competent mice (B6D2F1). First, an acute dose study was performed where established LLC tumors (400 – 600 mm^3) was treated with one dose of AG-013736 (100 mg.kg, PO) and tumor samples were collected and fixed for TUNEL and CD-31 co-immunostaining. The result showed that single dose of AG-013736 rapidly induced endothelial cell, but not tumor cell, apoptosis in the LLC tumor (Figure 3A). The apoptosis signal peaked at 4 – 7 hour postdose.

Multiple repeated-dose studies were carried out in the LLC model to evaluate anti-tumor efficacy of AG-013736. Figure 3B shows a representative dose-dependent tumor growth delay from one of the studies (DDH-123). AG-013736 treatment also reduced the amount of vessels in the tumor, as shown by the immunohistostaining of CD-31 in the tumor tissues (Figure 3C, left panels). In addition, tumor viability in AG-013736 treated tumors was also reduced compared to the vehicle-treated tumors (Figure 3C, right panels). Furthermore, high-dose AG-013736 (100 mg/kg, PO, BID) significantly enhanced animal survival as a result of delaying the spread and growth of metastatic tumors in the lung of LLC-bearing mice (Figure 3D, Study DDH-DCH-201).

Figure 3. Anti-tumor Efficacy by AG-013736 in Lung Carcinoma Models

A. Tumor Vessel Underwent Apoptosis after a Single Dose of AG-013736

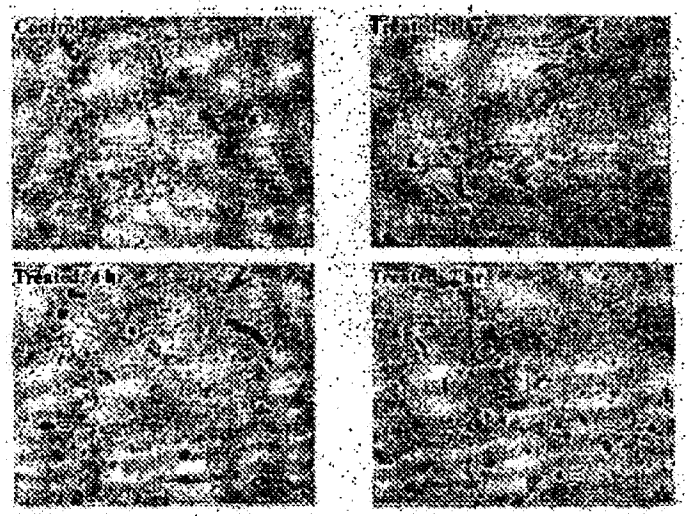
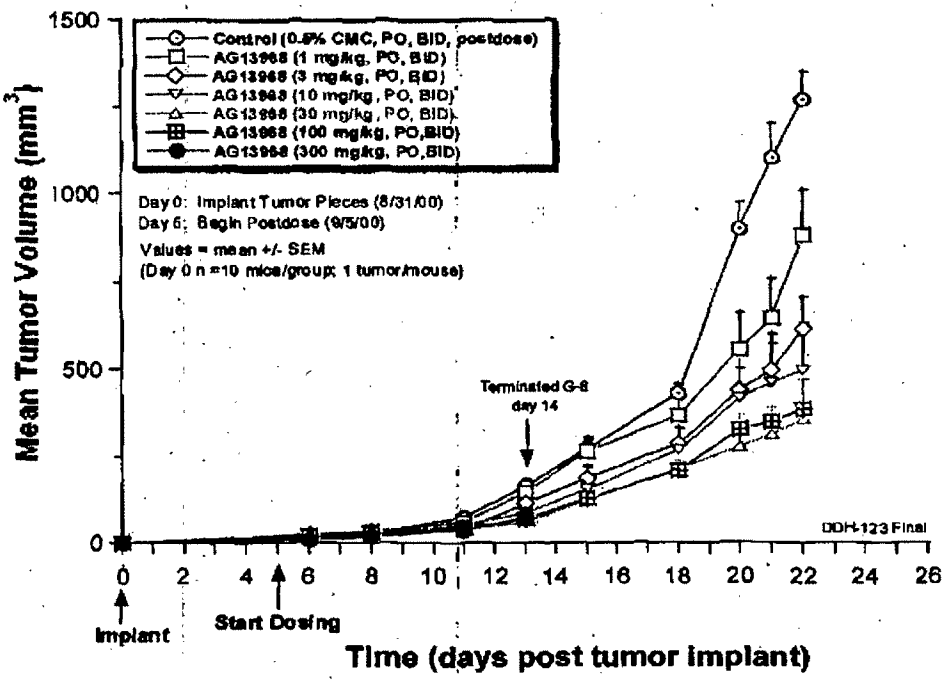


Figure 3. Anti-tumor Efficacy by AG-013736 in Lung Carcinoma Models (continued)

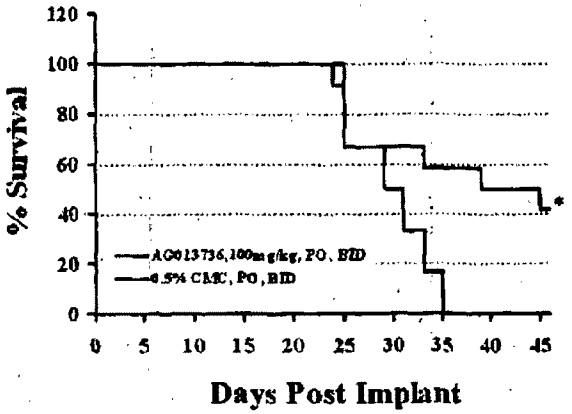
B. Dose-dependent Anti-tumor Efficacy by AG-013736 in the LLC Model



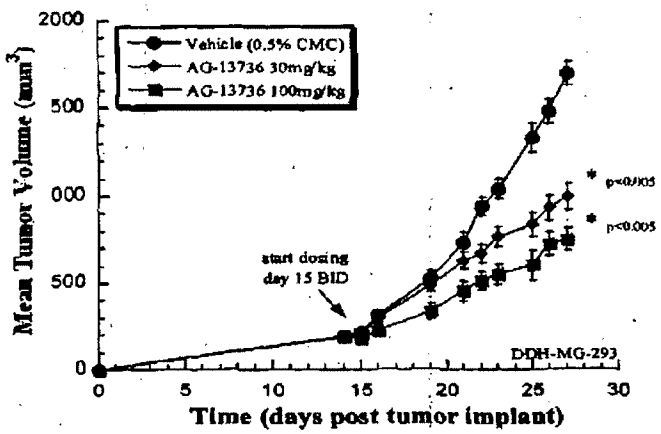
C. Reduction of CD-31 Staining and Tumor Viability by AG-013736

Figure 3. Anti-tumor Efficacy by AG-013736 in Lung Carcinoma Models (continued)

D. AG-013736 Increased Survival of LLC-bearing Mice (Kaplan-Myer *Plot*)



E. Anti-tumor efficacy in NCI-H526 SCLC model



AG-013736 significantly inhibited human lung tumor xenograft growth, angiogenesis in mice. A. Acute dose of AG-013736 rapidly induced apoptosis in the LLC tumor vessels; B. Dose-dependent TGI by repeated dose of AG-013736 in the LLC model; C. Anti-tumor efficacy was associated with reduction in CD-31 (left panels) staining and angiogenesis, and decrease in tumor cell viability (right panels). D. A Kaplan-Myer plot shows AG-013736 treatment improved the survival of LLC-bearing mice. E. Anti-tumor efficacy of AG-013736 in a SCLC model of NCI-H526. *See Methods for more information on tumor models and studies.*

Anti-tumor activity in the NCI-H526 model: AG-013736 was efficacious in the human SCLC model NCI-H526 (Figure 3E). At the dose of 30 mg/kg or 100 mg/kg, PO, BID, AG-013736 treatment produced a 47% and 61% TGI at 100 mg/kg, respectively. Both were statistically significantly different from the control group ($P < 0.05$).

6.1.4. Single Agent Anti-tumor Efficacy in Human Melanoma Models

Anti-tumor efficacy against established A375 melanoma tumor: The A375 human melanoma model was used to further evaluate the activity of AG-013736 against large and established tumors (Study DDH-MG-268). A735 tumors were established via sc implantation and the treatment was initiated when average tumor size reached about 350 mm^3. AG-013736 treatment at 10 and 30 mg/kg resulted in dose-dependent growth delay of 38% and 59%, respectively, compared to the vehicle-treated control tumors (Figure 4A). Although somewhat less than what was typically observed from studies where dosing was initiated when tumors were smaller, anti-tumor efficacy of AG-013736 at 30 mg/kg dose level against the established A357 tumors was statistically significant compared to the control group ($p = 0.035$).

Studies in the M24met melanoma model: M24met human melanoma model is an aggressive human melanoma that spontaneously metastasizes to lymph nodes and lungs in mice. The model was used frequently in the laboratory because it offered the possibility of evaluating multiple efficacy end points including primary tumor growth, metasasis, and survival of animals. Removing the primary tumors promoted the metastasis potential. Also important is that the M24met cells express mutant p53 and are reported to express high levels of oncogenic molecules such as N-RAS, ERK, as well as a5b1-integrins that are involved in tumor migration, adhesion and survival;[12] the cells do not express functional VEGFR but a low level of phospho-PDGFRs (Table 1).

First investigated was whether AG-013736 could inhibit the growth of primary M24met tumors implanted subcutaneously in nude mice (Study DDH-163). At dose levels of 3, 10, and 30 mg/kg, AG-013736 (PO, BID) significantly delayed tumor growth (Table 1) with TGIs of 60%, 49%, and 73%, respectively. Statistically, TGIs for the 3 and 10 mg/kg AG-013736 groups were not different from each other ($P = 0.4$), whereas the TGI in the group receiving 30 mg/kg AG-013736 was significantly greater than that of the 10 mg/kg dose group ($p = 0.033$).

The second investigation determined whether AG-013736 could inhibit metastasis when M24met was intradermally implanted in SCID (Balb/c) mice. The M24met cells (2.5×10^6) were implanted intradermally in the lower right flank of SCID mice. When the primary tumors reached the size of 300 – 400 mm^3 (usually ~ 2 weeks after implantation), they were surgically removed to promote distant metastasis. For the "Early Treatment" arms, the dosing of AG-013736 (50 mg/kg, PO BID) started one week prior to the primary tumor removal. For the "Late Treatment" arms, the treatment started 1 day before the primary tumor removal. The total treatment time was 3 weeks for all groups. At the end of the study, the metastatic tumors in the lymph nodes (mainly in the ipsilateral and counter-ipsilateral sites, with occasional tumors found in the ingroinal sites) were weighed and stained for CD-31 by IHC. Lungs were

fixed in the Bouin solution and metastatic tumors were subsequently manually scored under the microscope by four independent scientists. The results were averaged and are shown in Figure 4B (quantification of lymph node tumors) and Figure 4D (quantification of lung tumors). The study showed that AG-013736 significantly reduced metastatic incidence in the lymph nodes ($p < 0.0001$) and the lung ($p = 0.006$) for the early-dose group. For the late-dose groups, metastasis to the lymph nodes was also significantly and equivalently inhibited ($p < 0.0001$); the inhibition of lung metastasis was significant ($p = 0.04$) but was attenuated when compared with the early-dose group. Figure 4C and Figure 4E are representative tumor images from the vehicle control and AG-013736-treated lymph nodes and lungs of mice that were in the "Early Treatment" groups. The prevention therapy with the "Early Treatment" group appeared to have produced greater anti-metastasis activity in both the lymph node and lung tissues than did the "Late Treatment" group of the compound. AG-013736 treatment also resulted in decreased microvessel density (MVD) in lymph node tumors as measured by the IHC staining for CD-31 (Figure 4F).

Figure 4. AG-013736 Significantly Inhibited Human Melanoma Tumor Growth and Metastasis in Mice

A. Anti-tumor efficacy in established A375 model

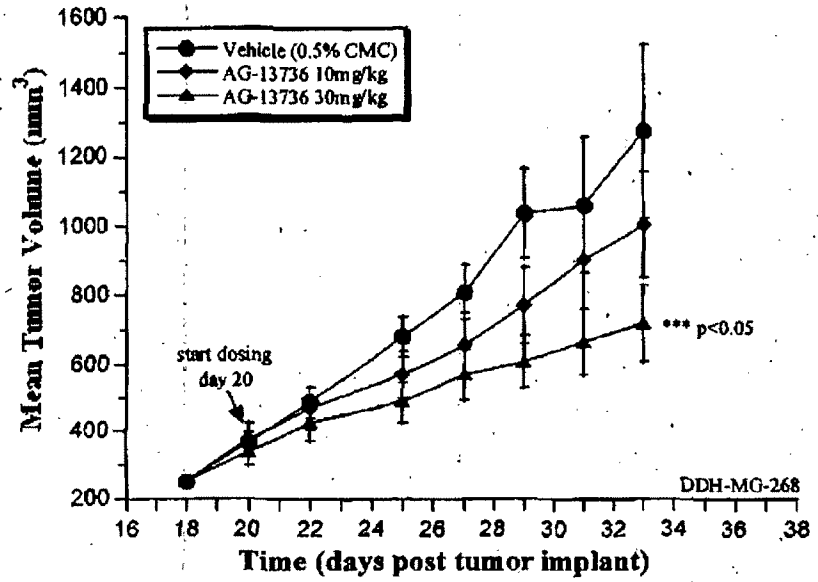
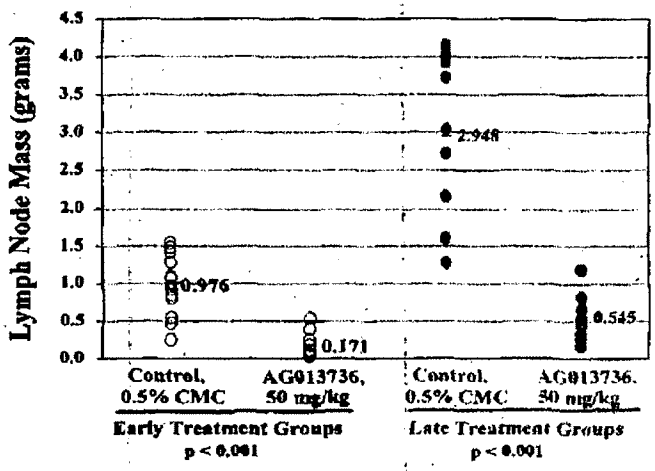
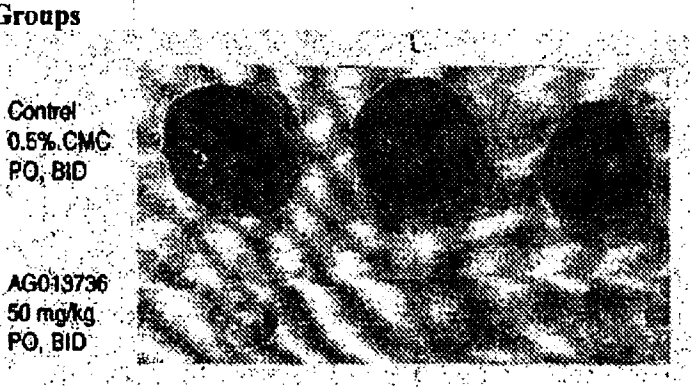


Figure 4. AG-013736 Significantly Inhibited Human Melanoma Tumor Growth and Metastasis in Mice (continued)

B. Inhibition of Lymph Node Metastasis by AG-013736 in M24met Model



C. Images of Lymph Node Metastatic Tumors in Control and AG-013736-Treated Groups



D. Inhibition of M24met Metastasis to the Lung by AG-013736

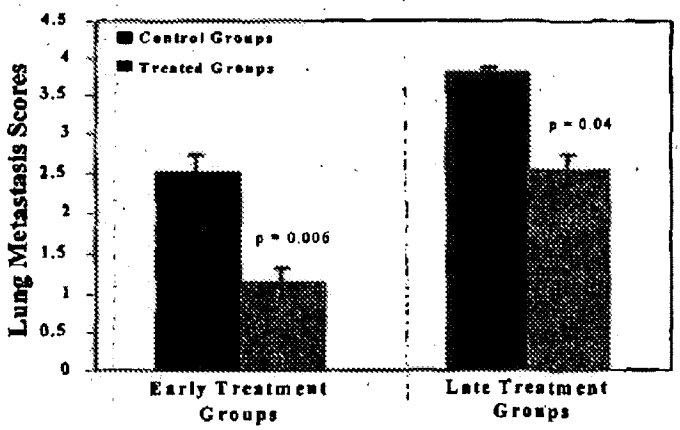
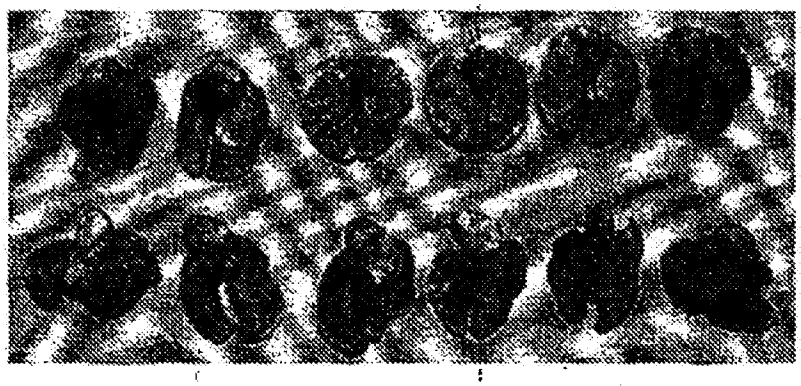
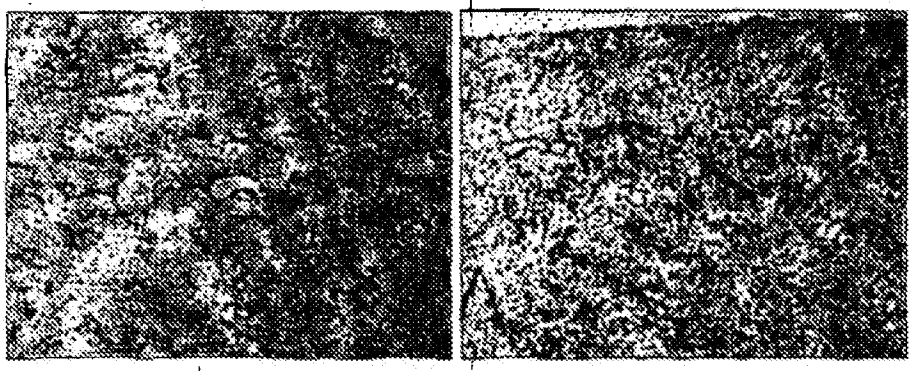


Figure 4. AG-013736 Significantly Inhibited Human Melanoma Tumor Growth and Metastasis in Mice (continued)

E. Images of Lung Metastatic Tumors in the Control and Early Treatment Groups



F. CD-31 Staining in Lymph Node Tumors of Control and AG-013736-treated Groups



Anti-tumor efficacy of AG-013736 in human melanoma xenograft tumor models. A. Anti-tumor efficacy against established A375 tumors by AG-013736. The treatment was initiated when the tumor reached ~ 350 mm^3 and dose-dependent growth inhibition was observed. B. Comparison of lymph node tumor weights in control and AG-013736 treated groups in the M24met tumor model. Results of both the early and late treatment groups are shown. C. Image of lymph node metastatic tumors in the control (top) and the AG-013736 treated (bottom) groups. D. Comparison of lung metastasis scores in the control and AG-013736 treated groups. Results of both the early and late treatment groups are shown. E. Image of lung metastatic tumors in the control (top) and the AG-013736 treated (bottom) groups. F. IHC staining for CD-31 (brown staining) in lymph node metastatic tumors, which demonstrated decreased microvessel density in the AG-013736 treated tumors (right) compared to the control tumors (left). Magnification = 10x.

6.1.5. Single Agent Anti-tumor Efficacy in Human Renal Carcinoma

SN12C-GFP renal carcinoma model: AG-013736 was investigated for its anti-tumor activity in GFP-transfected human renal carcinoma tumors that were orthotopically transplanted and grown in the kidneys of mice. Two days after tumor transplantation, AG-013736 was administered at 10, 30, and 100 mg/kg dose levels via the PO, BID regimen for either 42 days or 72 days. The green fluorescent signal of the tumor was measured intermittently throughout the treatment period and whole body optical images of GFP-expressing metastases were acquired at the end of the study. Table 2 summarizes the results of the study. Treatment with AG-013736 at 30 and 100 mg/kg, but not 10 mg/kg, clearly resulted in statistically-significantly smaller primary tumor mass than the vehicle control on both termination days ($p < 0.05$). TGIs from the 10, 30, and 100 mg/kg dose groups were 54%, 56%, and 63% for the 41-day treatment arm, respectively; TGIs from these dose groups were 61%, 74%, and

70% for the 72-day treatment arm, respectively. In conclusion, AG-013736 at 30 mg/kg and 100 mg/kg significantly inhibited the growth of orthotopic human renal cancer SN12C-GFP in mice. This pilot study with only 5 mice per group was not designed to evaluate metastasis, which would require 20 animals per group.

Table 2. Summary of Treatments and TGI in Orthotopic RCC Model of SN12C-GFP

Group						
Group 1, Control	0.99 (0.43)	-	-	2.43 (1.58)	-	-
Group2, AG-013736, 10 mg/kg	0.45 (0.14)	0.057	54.5	0.95 (0.5)	0.082	60.9
Group 3, AG-013736, 30 mg/kg	0.44 (0.06)	0.048	55.6	0.63 (0.28)	0.037	74.1
Group 4, AG-013736, 100 mg/kg	0.37 (0.05)	0.03	62.4	0.73 (0.25)	0.045	70

Detailed study information and results are included in the study report[7].

6.1.6. Single Agent Anti-tumor Efficacy in Human and Rat Glioblastoma

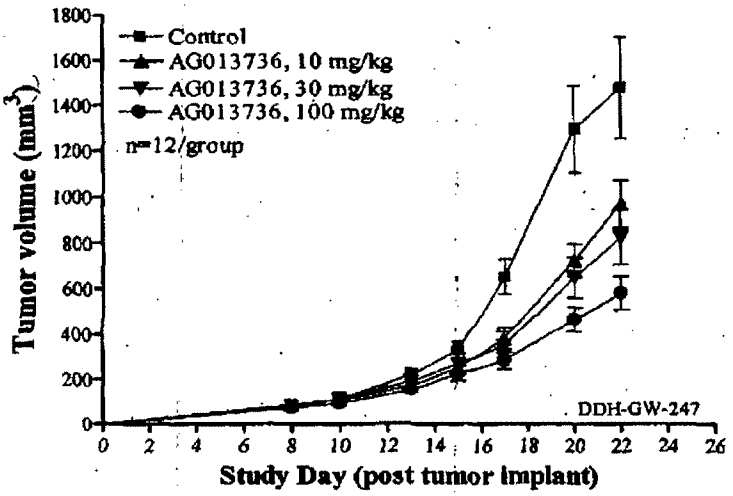
Glioma tumors are known to be highly vascularized when grown as xenografts in animals and the cells often express certain functional RTKs including PDGFRs. The U87MG and C6 glioma tumor expression of the phosphorylated and unphosphorylated proteins of PDGFR-α and β was confirmed (Table 1). In vitro assays showed that AG-013736 potently inhibited PDGF BB-mediated U87MG migration in culture flasks.2 Furthermore, acute dosing of AG-013736 partially (30 mg/kg dose level) or nearly completely (100 mg/kg dose level) inhibited PDGFR-β phosphorylation in C6 tumors.3 Subcutaneous xenograft tumors from both glioma tumor lines were established to evaluate anti-tumor efficacy of AG-013736 in these models.

Anti-tumor activity in the C6 rat glioma model: In study DDH-GW-247, AG-013736 treatment (PO, BID) resulted in dose-dependent growth delay of C6 tumors in nude mice (Figure 5A). A 37%, 47%, and 64% TGI was observed for 10, 30, and 100 mg/kg dose levels, respectively.

Anti-tumor activity in the U87MG human glioblastoma model: AG-013736 treatment (PO, BID) in the U87MG tumor study DDH-GW-273, resulted in dose-dependent growth delay of U87MG tumors in nude mice (Figure 5B). TGIs of 44%, 69%, and 74% were observed for the 10, 30, and 60 mg/kg AG-013736 dose levels, respectively.

Figure 5. Inhibition of Glioma Xenograft Tumor Growth by AG-013736

A. Anti-tumor Efficacy in C6 Glioma Model



B. Inhibition of U87MG Tumor Growth by AG-013736

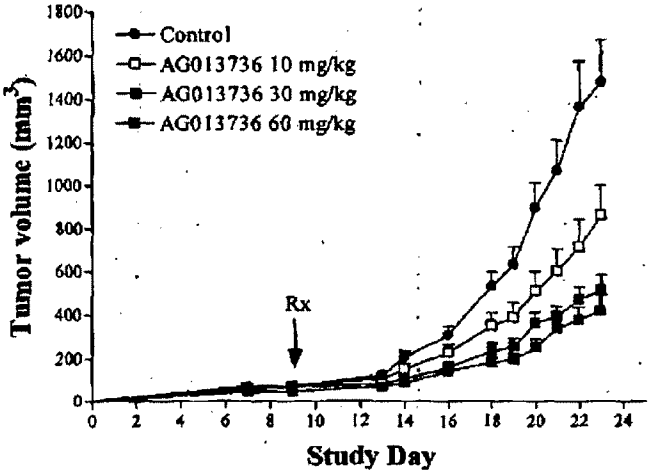
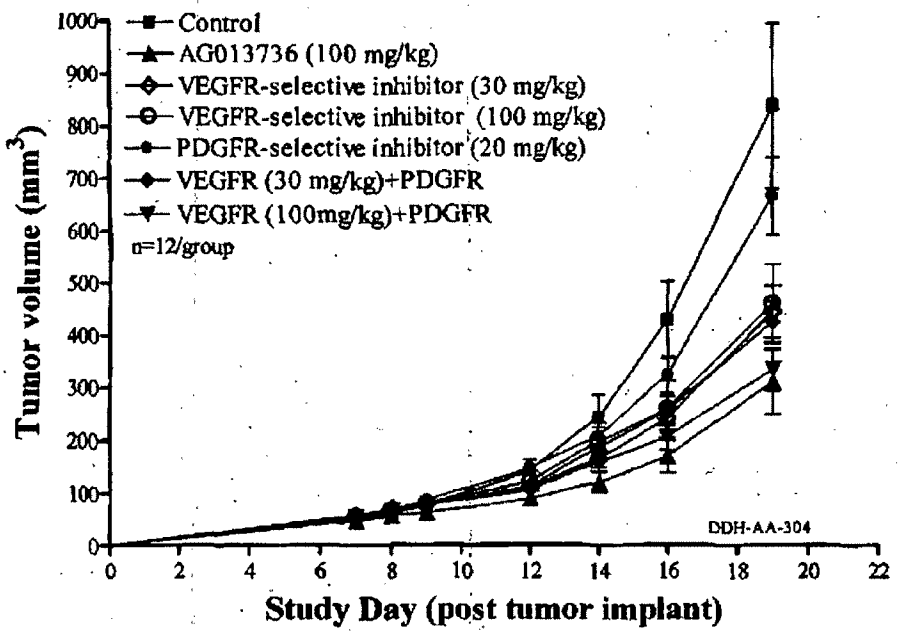


Figure 5. Inhibition of Glioma Xenograft Tumor Growth by AG-013736 (continued)

C. AG-013736 Single Agent Activity was Equivalent to Efficacy from Combination of VEGFR- and PDGFR-selective Inhibitors



Anti-tumor activity of AG-013736 in human and rat glioma models. A.) Dose-dependent inhibition of rat C6 glioma tumor growth in mice; B.) Dose-dependent inhibition of human U87MG glioblastoma tumor growth in mice; C. High-dose AG-013736 (100 mg/kg) produced anti-tumor efficacy similar to that from the combination of high-dose VEGFR-selective inhibitor and PDGFR-selective inhibitor in the U87MG model.

In addition to inhibition of VEGFRs, nanomolar concentrations of AG-013736 have been shown to inhibit PDGFR targets in endothelial and glial tumor cells in vitro. In vivo, it was previously shown that high dose of AG-013736 (100 mg/kg) produced a near complete inhibition of PDGFR-β phosphorylation in the C6 glioma model that was associated with plasma exposure exceeding PDGFR inhibition for at least 8 hours/day.[3] Thus, it was recognized that against PDGFR-positive tumor cells, a high dose level of AG-013736 may have a dual mechanism of action through the inhibition of angiogenesis, mediated by VEGFR antagonism, and direct inhibition of tumor cell growth through PDGFR blockade. This hypothesis was tested through the use of highly selective VEGFR and PDGFR pharmacological antagonists (all PGRD research compounds) in the U87MG human glioblastoma model that expresses functional PDGFR-α and PDGFR-β (Table 1).

AG-013736 (100 mg/kg, PO, BID) demonstrated the highest TGI in comparison with the more selective antagonists as single agents (Figure 5C). The combination of VEGFR-selective (high dose) and PDGFR-selective compounds together yielded TGI equivalent to that of AG-013736 alone. These results are consistent with those from a previous study (DDH-GW-

297, not shown). These observations suggest that high-dose AG-013736, in this case, 100 mg/kg, was capable of inhibiting both VEGFRs and PDGFRs in vivo and achieving the same magnitude of anti-tumor efficacy as from combined therapy using both VEGFR and PDGFR inhibitors. Therefore, the simultaneous inhibition of both VEGFRs and PDGFRs is an important component of AG-013736 mechanism of action in this model.

6.1.7. Single Agent Anti-tumor Efficacy in Human Non-Hodgkin's Lymphoma Model

Namalwa NHL model: The Namalwa cell line was reportedly one of the most aggressive NHL lines regarding the efficiency of engraftment (IP or sc), tumor angiogenesis, and progression that was highly correlative to VEGF production by the engrafted tumor. IP-engrafted Namalwa tumors reportedly had similar disease characteristics as human NHL[13,14] Due to these features, the model has been used for assessing anti-tumor efficacy of anti-angiogenic agents (endostatin) and cytotoxics agents, as well as assessing circulating endothelial cells (CECs) as a biomarker for therapeutic agents. AG-013736 was tested for its anti-tumor activity in this model. Separately, AG-013736 was also evaluated for its ability to modulate the amount of CECs in the Namalwa tumor-bearing mice at the European Institute of Italy (F. Bertolini).

The Namalwa model characteristics are that the tumor grows as a soft solid mass in the IP cavity and tumor ascites accumulate over time along with local tumor spreading and invasion. The animals quickly die of local and distant metastases. In the DDH-RF-240 study, AG-013736 was dosed at 50 mg/kg, PO, BID starting one day after IP implantation of the tumor cells. The gross tumor burden was assessed by measuring animal body weight gain over the time course of the study. AG-013736 treatment significantly reduced the body weight gain of the mice ($P < 0.05$, Figure 6A). Figure 6B shows AG-013736 treatment reduced tumor mass in the IP cavity of mice compared to vehicle-treated mice. AG-013736 treatment also significantly reduced the viable KIT-positive cell population (Figure 6C).

Figure 6. Reduction of Tumor Burden in Namalwa Model of NHL by AG-013736

A. AG-013736 Treatment Suppressed Body Weight Gain in Human Namalwa Model

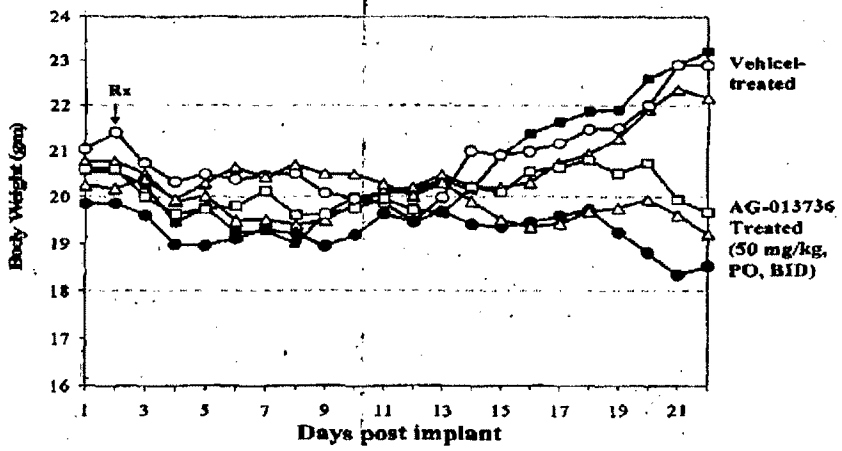
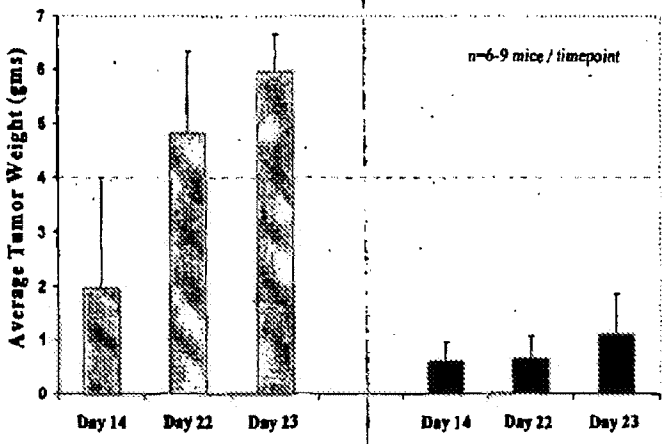


Figure 6. Reduction of Tumor Burden in Namalwa Model of NHL by AG-013736 (continued)

B. AG-013736 Treatment Decreased Tumor Burden in the IP Cavity of Mice Bearing the Human Namalwa Lymphma Tumors



C. AG-013736 Treatment of Namalwa Tumors Reduced KIT$^+$ Population in Peripheral Blood of Mice

	CONTROL	AG-013736	p value
DAY 14	0.02% ± 0.04	0.67% ± 0.4	0.05
DAY 22	0.55% ± 0.12	0.046% ± 0.2	0.00004
DAY 23	2.24% ± 0.7	0.32% ± 0.1	0.008

Anti-tumor efficacy of AG-013736 in human lymphoma model of Namalwa. A. Treatment with AG-013736 (50 mg/kg, PO, BID) significantly suppressed body weight gain due to tumor and ascite burden in NOD/SCID mice. The body weight difference between AG-013736 treated groups and vehicle-treated groups were statistically different ($P<0.05$). B. Tumors in the IP cavity of the mice were collected and weighed on the sacrifice days (day-14, 21 and 22 post initiation of the treatment). AG-013736-treatment produced significant reduction in tumor burden in the mice ($P < 0.05$). C. Peripheral blood cells were collected and processed for PBMNCs. The FACS analyses were performed using antibodies that detected viable cells that were 7-AAD$^-$ and KIT (CD117+) positive.

In separate studies using the Namalwa model, daily administration of AG-013736 (10 - 30 mg/kg, IP) generated a significant delay of lymphoma onset and reduction of lymphoma growth in comparison to control mice ($p<0.0001$). Mice treated with daily AG-013736, a maximum tolerable dose (MTD) of cyclophosphamide (CTX), or PBS as a control were evaluated every 5 days for the presence of mature circulating endothelial cells (CEC) and circulating endothelial progenitors (CEP). The first course of MTD CTX delayed but did not prevent tumor growth, and a dramatic increase in CEP number and viability was observed a few days after treatment with CTX. When CTX-treated animals were switched to

be given daily dosing of AG-013736, CEP mobilization was significantly down-regulated and both CEP viability and tumor recurrence were significantly reduced ($p<0.001$) compared to PBS-treated mice. Thus, AG-013736 had anti-tumor activity that was associated with reducing the mobilizaton of CEP associated with the MTD treatment of cytotoxics agent cyclophosphamide. This provides a strong rationale for combining AG-013736 with chemotherapy in the clinic. Detailed study design and results can be found in S. Paul et al.[15]

6.2. Dose Frequency Studies

To study how to maximize the anti-tumor efficacy of AG-013736, several types of dose scheduling studies were carried out, including dose frequency studies, i.e., BID versus SID, BID versus continuous infusion, intermittent dosing ("On/Off" studies), and tumor regrowth studies.

6.2.1. BID versus SID Dosing and Daily Dosing versus Intermittent Dosing in MV522 Tumor Model

Anti-tumor efficacy of AG-013736 was evaluated following either BID (30 mg/kg) or SID (60 mg/kg) dosing in the MV522 model. In the same study, TGI by continuous daily dosing (BID or SID) versus intermittent dosing (BID or SID) was also explored. This was to assess whether short dosing breaks (5:2 or 4:3 schedule) would show a statistically significant difference in anti-tumor efficacy compare to continuous daily dosing. All groups had an initial loading dose period before dosing breaks were implemented. The study design is shown in Table 3.

Table 3. Design and Efficacy Outcome of Dose Scheduling Study (DDH-KA-248)

Group#	BID or SID	Group Names	Description	TGI%	Stats Compared to Grp2*
1	BID	No dose	No dose	0	<0.01
2		Daily dosing schedule	Daily BID	82	--
3		(7 + 5:2) schedule	BID daily x 7 days then (M-F) x 3wk	76	0.41
4		(14 + 5:2) schedule	BID daily x 2wk, then (M-F) x 2wk	74	0.16
5		(13 + 4:3) schedule	BID daily x 13 days, then (M-Thur) x 2wk	68	0.08
6		(7 + 4:3) schedule	BID x 7 days , then (M -Thur) x 3wk	66	0.04
7	SID	Daily dosing schedule	Daily SID	76	0.34
8		(7 + 5:2) schedule	SID x 7 days, then (M-F) x 3wk	65	0.02
9		(14 + 5:2) schedule	SID daily x 2wk, then (M-F) x 2wk	69	0.25

* Statistics were obtained from Student t-Test based on data from the last day of study (day 33).

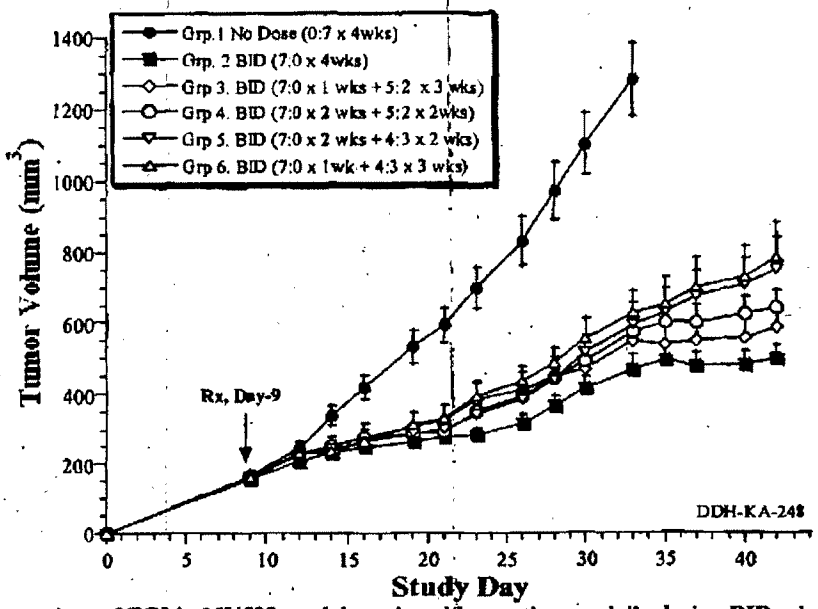
All AG-013736 dose schedules produced significant anti-tumor efficacy compared to the No-dose control group. Among the BID dosing groups, the continuous daily dosing (Group 2) generated the greatest anti-tumor efficacy (82%). TGI (68%) in Group 6 was statistically significant compared to Group 2 ($P = 0.04$). Group 6 received the shortest loading dose period

(7 days) before it was switched to the 4-day on/3-day off schedule for the rest of the study (33 days). All other BID dosing groups did not exhibit statistically-significantly less TGI compared to Group 2, although there appeared to be an inverted correlation between the length of loading dose period, dose breaks, and the magnitude of TGI (Figure 7.). Similarly, this trend was also observed among the SID dose regimen groups; both groups on dosing break schedules had less TGI compared to the continuous daily SID dosing group (Group 7). The differences between the TGI among the SID dosing groups was not significant statistically.

Between the paired SID and BID dosing groups, TGI produced by daily SID dosing of AG-013736 at 60 mg/kg (76%) was not different statistically from that produced by daily BID dosing of AG-013736 at 30 mg/kg (82%).

In conclusion; in this 33-day dosing study using one total daily dose, treatment of MV522 tumors with 30 mg/kg, BID or 60 mg/kg, SID produced similar anti-tumor efficacy; TGI by BID regimen proved better than the SID regimen. Overall, short dosing breaks (weekends, holidays) following a loading dose treatment period did not significantly negatively affect TGI, although varying degree of was observed that was dependent upon the length of dosing breaks and the length of loading dose treatment period.

Figure 7. TGI Produced by BID Dosing with or Without Dosing Breaks



Comparison of TGI in MV522 model produced by continuous daily dosing BID schedule versus dosing break BID schedule of AG-013736. The continuous BID daily dosing schedule (Group 2, blue filled square) generated the greatest anti-tumor efficacy. Varying degree of compromise in TGI was observed in the dosing break BID groups compared to Group2. Only Group 6 was statistically significantly different from Group 2 by the Student t-Test analysis.

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Kinase inhibitors in the treatment of renal cell carcinoma

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Contents

Abstract

Immunotherapy confers a small but significant overall survival advantage in metastatic renal cell carcinoma (RCC) but a need exists to develop more effective systemic therapies. Angiogenesis has a key role in the pathophysiology of renal cell carcinoma and vascular endothelial growth factor (VEGF) is an important mediator of this process. Sunitinib, sorafenib and axitinib are new agents which belong to a class of drugs called kinase inhibitors and inhibit the VEGF, platelet-derived growth factor (PDGF) and c-KIT receptor tyrosine kinases. Temsirolimus inhibits the mammalian target of rapamycin (mTOR). All these agents have shown significant activity with manageable toxicity in metastatic RCC in phase 2 studies in patients generally pretreated with immunotherapy, whilst prolonged progression-free survival in a phase 3 study

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has been reported with sorafenib in comparison with placebo. Further phase 3 trials are recruiting and the combination of kinase inhibitors with other therapies is under investigation.

Keywords: Renal cell carcinoma; Metastatic; Systemic therapy; Kinase inhibition; Targeted drugs

1. Introduction

1.1. Epidemiology and pathology of renal cell carcinoma

Carcinoma of the kidney accounts for 2–3% of all cancers [1,2] and increased in incidence by almost 20% in the UK between 1991 and 2000 [Cancer Research UK CancerStats Monograph 2004]. Approximately two thirds of patients with renal cell carcinoma (RCC) present with disease localized to the kidney that can often be cured by surgery. This review will not further consider the surgical treatment of localized renal cell carcinoma.

Renal cell carcinomas have been classified histologically as clear cell (~60–80%), papillary (~10–15%), chromophobe (~5–10%), collecting duct and medullary (<1% each) [3–5]. Metastatic clear cell renal carcinoma is associated with a better outcome than either chromophobe or papillary histology [6].

1.2. Angiogenesis in metastatic renal cell carcinoma

Von Hippel-Lindau (VHL) disease is an autosomal dominant condition [7] characterized by a predisposition to haemangioblastomas of the retina and central nervous system (most commonly cerebellum and spinal cord) and to clear cell carcinoma of the kidney [8]. Individuals with VHL harbour one mutated version of the VHL gene and tumour development is associated with somatic mutation of the remaining wild-type allele. Von Hippel-Lindau disease is the commonest basis for inherited renal cell carcinoma but is also relevant to sporadic disease as inactivation of both genes occurs in approximately two thirds of cases. This inactivation is most commonly as a result of mutation [9–11] but can also occur as a result of promoter hypermethylation [9–14].

The Von Hippel-Lindau protein is involved with cellular responses to hypoxia. In normoxic conditions the VHL protein binds to hypoxia inducible factor-1α (HIF-1α) and HIF-2α which as a consequence become ubiquinated and tagged for degradation in the proteasome [15]. In hypoxic conditions or in the absence of VHL, HIF-1α accumulates; this stimulates the production of growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor α (TGFα, which binds to EGFR) and platelet-derived growth factor (PDGF) (Fig. 1). These molecules act in a paracrine loop on their cognate receptor tyrosine kinases, stimulating cell proliferation and angiogenesis. This may account for the typical vascularity of renal cell carcinomas and may explain the clinical benefits reported with the anti-VEGF monoclonal antibody bevacizumab in this disease [16,17]. There is also a clear theoretical rationale for the investigation of small molecule inhibitors of receptor tyrosine kinases regulated by VHL and inhibitors of HIF-1α production in renal cell carcinoma [18] (Table 1).

1.3. Treatment of metastatic renal cell carcinoma

A third of patients present with disseminated disease and a further third of patients treated with curative intent for localized disease subsequently relapse. Metastatic renal cell carcinoma is incurable and the intent of treatment is palliative. The prognosis for metastatic RCC is poor with a median survival of 10–12 months [19–22].

1.3.1. Surgery

Surgery to remove the primary tumour in metastatic RCC results in a survival benefit when interferon is administered post-operatively. This has been demonstrated in two randomized trials, European Organization Research and Treatment of Cancer (EORTC) 30947 [23] and Southwest Oncology Group (SWOG) 8949 [24]. Both trials recruited patients with performance status 0 or 1 who were randomized to nephrectomy followed by standard treatment with interferon-α versus treatment with interferon-α alone. Median survival increased from 7 to 17 months and from 8 to 11 months, respectively, in the surgery groups.

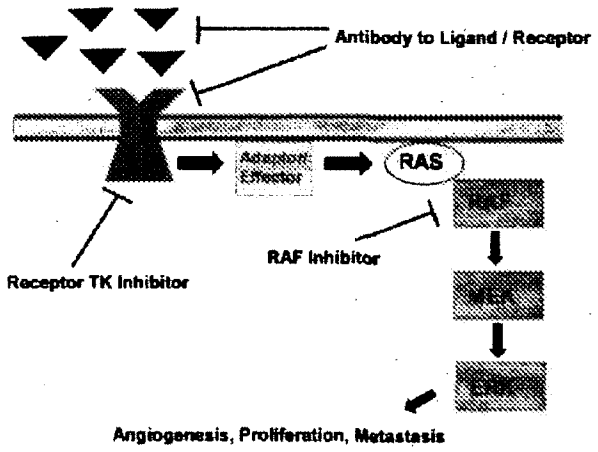

Fig. 1. Ligand binding to a receptor tyrosine kinase induces oligomerization and autophosphorylation of the cytoplasmic domain and contingent increased TK activity. Multiple downstream intracellular signaling pathways such as RAS/RAF/MEK/ERK may as a result be activated, resulting in proliferation, angiogenesis and metastasis. Potential therapeutic targets are illustrated.

Table 1
Tyrosine kinase inhibitors in clinical trials in renal cell carcinoma

Agent	Target	Trial Phase
Erlotinib (OSI-774)	EGFR	2
Gefitinib (ZD1839)	EGFR	2
Semaxinib(SU5416)	VEGFR1, VEGFR2	2
Sorafenib (BAY 43-9006)	VEGFR2, VEGFR3, PDGFR, FLT-3, c-KIT, CRAF, wtBRAF, V599E BRAF	2, 3
Sunitinib (SU011248)	VEGFR2, PDGFR, FLT-3, c-KIT	2, 3
Axitinib (AG-013736)	VEGFR1, VEGFR2, PDGFR, c-KIT	2
Temsirolimus (CCI-779)	mTOR	2, 3

1.3.2. Immunotherapy

Response rates to combination chemotherapy [25] and to hormonal agents [26] in metastatic RCC are 5–10% but immunotherapy with interferon (IFN) produces response rates of 10–20% with median response durations of 3–16 months [27]. Randomized controlled trials have shown a survival advantage with IFN therapy compared to non-immunotherapy [20,28] and a Cochrane review and meta-analysis has also recently confirmed the value of IFN-α in metastatic RCC [29]. It should be noted that for patients with intermediate prognosis metastatic disease [30], a randomized trial reported in abstract form (ASCO 2005, Abstract 4511, Negrier et al.) has shown that immunotherapy with either interferon-α or interleukin-2 or the combination of both drugs does not result in a survival benefit over medroxyprogesterone acetate.

In non-randomized trials approximately 9% of patients with metastatic RCC have a complete response (CR) to treatment with high dose intravenous interleukin-2 (HDIV IL-2) and in 70–80% of these patients the CRs are prolonged [31]. High dose intravenous interleukin-2 is the only therapy that results in durable complete responses but causes substantial toxicity. A randomized trial of HDIV IL-2 versus SC IL-2 + IFN-α reported a significantly higher response rate for HDIV IL-2 but no difference in median survival although for patients with bone or liver metastases or a primary tumour *in situ*, survival was significantly longer with HDIV IL-2 [32]. There may therefore be a role for high dose IL-2 in selected patients with metastatic RCC and good performance status. Recent data suggest that high tumour carbonic anhydrase IX expression is predictive of significantly prolonged median survival after IL-2 therapy [33] and this antigen therefore may be a useful biomarker. The current standard therapy for metastatic RCC is subcutaneous single agent IFN-α, given until the development of either unacceptable toxicity or progressive disease.

In summary, immunotherapy for metastatic RCC has poor response rates and significant toxicity but offers the possibility of prolonged complete remission or cure. Despite the low response rates to interferon, a survival benefit has been reported and this may be because disease stabilisation occurs in a significant number of patients. This is a beneficial outcome for patients and shows the value of time rather than response-based endpoints in clinical trials. This is particularly relevant to renal cell carcinoma as the disease can have a variable natural history and is also relevant to clinical trials of agents that are cytostatic rather than cytotoxic.

2. Kinase inhibitors

2.1. Introduction

The term 'kinase inhibitor' generally refers to small molecule drugs that inhibit tyrosine kinases (TKs). Tyrosine kinases are enzymes that catalyze the transfer of γ-phosphate groups from adenosine triphosphate (ATP) to the hydroxyl groups of tyrosine residues on target proteins. The phosphorylation of target proteins such as signaling molecules is generally an activating event that in tumours can cause increased cellular proliferation and growth, prevent apoptosis and promote metastasis and angiogenesis.

Tyrosine kinases can be classified as receptor and non-receptor kinases. Receptor TKs such as epidermal growth factor receptor (EGFR/ERBB1) span the cell membrane and transduce signals from the extracellular environment to the cell interior, whilst non-receptor TKs such as c-ABL relay intracellular signals. Receptor TKs are inactive, monomeric and unphosphorylated in the absence of ligand binding. Ligand binding induces oligomerization and autophosphorylation of the cytoplasmic domain and contingent increased TK activity. Multiple downstream intracellular signaling pathways [34] such as RAS/RAF/MEK/ERK, PI3K (phosphoinositol 3′-kinase) and protein kinase C (PKC) may then be activated (Fig. 2). Non-receptor TKs can be activated by various intracellular signals such as phosphorylation by other kinases.

Tyrosine kinases can be dysregulated in cancer cells in various ways. An example is BCR-ABL in CML which occurs as a resulted of the balanced (9;22) chromosomal translocation. This translocation results in the production of the non-receptor TK BCR-ABL fusion protein; a domain in BCR overcomes the autoinhibition of ABL, leading to constitutive TK activation. A second mechanism of TK dysfunction in cancer cells is via the overexpression of either a receptor TK or its ligand; an example is the overexpression of ligands such as vascular endothelial growth factor (VEGF), transforming growth factor α (TGFα) and platelet-derived growth factor (PDGF) in renal cell carcinoma. A further mechanism is via increased sensitivity of a receptor to its ligand; an example

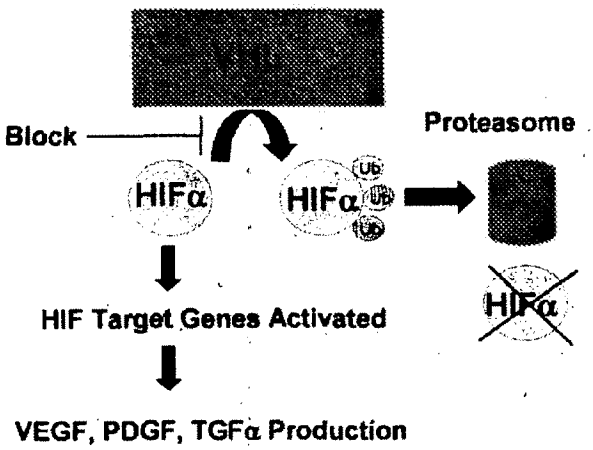

Fig. 2. In normoxia the VHL protein binds to hypoxia inducible factor α (HIF-α) which is ubiquinated and tagged for degradation in the proteasome. In hypoxia or in the absence of VHL, HIF-α accumulates, stimulating the production of growth factors such as VEGF, TGFα and PDGF. These molecules act on receptor tyrosine kinases, stimulating cell proliferation and angiogenesis.

of this is mutations in EGFR in some non-small cell lung cancers (NSCLCs) that alter receptor signaling [35,36].

Drugs that interfere with TK signaling are used in the treatment of many types of solid tumour. The two main classes of such drugs are monoclonal antibodies such as trastuzumab, cetuximab and bevacizumab and small molecules ('kinase inhibitors') such as imatinib, erlotinib, gefitinib, sorafenib (BAY 43-9006) and sunitinib (SU011248).

2.1.1. Kinase inhibition by monoclonal antibodies

Monoclonal antibodies against receptor TKs or their ligands disrupt TK signaling by preventing ligand binding and receptor internalization. For example, bevacizumab is directed against VEGF, an important regulator of the physiological and pathological process of angiogenesis [37,38]. Bevacizumab has been evaluated in metastatic RCC as monotherapy in a randomized phase II trial in which doses of 3 and 10 mg/kg every 2 weeks were compared with placebo [16]. Time to progression (TTP) and response rate were the primary endpoints; an interim analysis after the randomization of 110 patients reported a prolongation of TTP in the 10 mg/kg group (4.8 months versus 2.5 months, HR 2.55, $p < 0.001$). The trial was stopped early on the basis of this result although no survival benefit was demonstrated and there were only four responses to treatment (response rate 10%, all in the higher dose arm). Bevacizumab generally was tolerated well with asymptomatic proteinuria and hypertension the commonest side effects.

2.1.2. Kinase inhibition by small molecules

Small molecule drugs disrupt TK signaling by preventing the binding of either ATP or protein substrates. For example, imatinib inhibits the BCR-ABL fusion protein in chronic myelogenous leukaemia (CML) [39] and c-KIT (CD 117) in gastrointestinal stromal tumours (GISTs) [40] and has revolutionized the management of these diseases. The tyrosine kinase inhibitors gefitinib and erlotinib have been used in the treatment of non-small cell lung cancer (NSCLC) but neither drug, when used in combination with standard cytotoxic chemotherapy as first-line therapy, showed a benefit over chemotherapy alone [41,42]. Both drugs have shown modest activity as single agents in NSCLC [43,44] but the clinical benefit is smaller than that seen with imatinib in CML or GIST and may be limited to specific subgroups of patients [35,45,46] that demonstrate increased sensitivity to kinase inhibition such as female non-smokers with adenocarcinomas. These data emphasise the potential importance of patient selection for treatment with kinase inhibitors.

This remainder of this review will focus on the small molecule kinase inhibitors that have generated the most promising safety and efficacy data in clinical trials in renal cell carcinoma, classified according to their mechanism of action.

2.2. The epidermal growth factor receptor (EGFR)

2.2.1. EGFR signaling in metastatic renal cell carcinoma

The epidermal growth factor receptor (EGFR) is overexpressed in approximately 70% of renal cell carcinomas [47] and as a result there has been interest in this receptor tyrosine kinase as a therapeutic target in this disease. Clinical trials of both antibodies to EGFR [48] and of the orally administered small molecule EGFR kinase inhibitors gefitinib and erlotinib (Table 1) have been conducted. The data reported for gefitinib and erlotinib are reviewed here.

2.2.2. Gefitinib (ZD1839) and Erlotinib (OSI-774)

Three phase 2 studies of gefitinib in the treatment of metastatic renal cell carcinoma have been published [49–51]. A total of 67 patients were included in these studies and there were no responses to treatment. Some patients had stable disease on treatment but the relevance of this is unclear in the absence of a control group. The trial reported from the the Memorial Sloan Kettering Cancer Center (MSKCC) [50] retrospectively compared time to progression (TTP) on gefitinib with TTP in patients who received interferon-α therapy from the MSKCC renal cell carcinoma database. Historical controls treated with interferon-α had a median TTP of 4.7 months, with 40% of patients having disease progression at 4 months. During treatment with gefitinib, 81% of patients had progression of disease within 4 months of the start of therapy. Gefitinib appears therefore to lack significant activity in this setting.

Erlotinib has been combined with bevacizumab in a phase 2 trial in metastatic renal cell carcinoma [17]. Sixty-three patients were treated, 68% of whom had had no prior systemic therapy. Fifty-nine patients were evaluable for response of whom 15 (25%) had objective responses (14 partial responses and 1 complete response). Thirty-six patients (61%) had sta-

ble disease and 13 of these pts had minor objective responses (10–30% decrease in tumour size by RECIST criteria [52]). The median progression-free survival was 11 months and median survival had not been reached at the time of reporting. Toxicity was generally manageable although there was 1 grade 4 toxicity (a gastrointestinal bleed). Over 10% of patients also had grade 3 diarrhoea, rash, nausea or vomiting. The high response rate reported with this combination was surprising given the lack of response to gefitinib and the low response rate to bevacizumab [16] as a single agent.

2.3. Multitargeted kinase inhibitors

2.3.1. Semaxinib (SU5416)

The receptor tyrosine kinases VEGFR1 and VEGFR2 are inhibited by semaxinib (Table 1). Semaxinib has been studied as a single agent in a phase 2 trial in metastatic RCC in 29 patients who were treated intravenously twice weekly [53]. There were no responses by RECIST criteria but 1 patient had a minor response and 5 patients had stable disease of 24 evaluable patients.

Semaxinib has also been studied in combination with interferon-α therapy in a phase 2 trial [54]. Thirty patients were treated, 23 of whom had not had prior systemic therapy. There were no complete or partial responses. Fifteen of the 23 evaluable patients had stable disease after 3 months on the study. Significant toxicity was reported with this regimen: 13 of 30 evaluable patients had grades 3 or 4 lymphopaenia and 14 of 30 had grades 3 or 4 fatigue. At least 10% of patients also had grades 3 or 4 dehydration, headache, hyperglycaemia, nausea and vomiting, infection, transfusion requirements or leucopaenia. There were 3 on-study deaths, 2 of which were infection-related. Semaxinib was given twice weekly with pulsed steroids, the latter to prevent allergic reactions as it is soluble only in Cremophor. The authors of the study speculate that pulsed steroids may have contributed to the regimen's toxicity, including susceptibility to infection. Furthermore, the use of steroids could have attenuated the antiangiogenic and immunomodulatory effects of interferon.

Given that semaxinib inhibits VEGFR1 and VEGFR2, it is noteworthy that no responses to therapy have been seen with this drug and that the response rate to the anti-VEGF monoclonal antibody bevacizumab as a single agent in metastatic RCC is only 10% [16]. However, in the trial of bevacizumab versus placebo, a highly significant prolongation of time to progression (TTP) was seen in the treatment group. The design of the phase 2 trials of semaxinib prevents conclusions being drawn about time to progression and highlights one of the problems of designing phase 2 trials of cytostatic agents. The explanation for the lack of efficacy of this drug as measured by response rate is not clear but is intriguing in the light of the bevacizumab data. One possibility is that the TK targets are not being 'hit' at the dosing and scheduling of semaxinib used. This contention is supported by a study [55] that measured the effect of semaxinib (and SU6668, a 2nd generation derivative) on downstream markers of angiogenesis inhibition in matched pre- and post-treatment biopsies obtained in three clinical trials. The biological activity observed in patient tumour samples with both drugs was lower than that associated with growth inhibition of xenografted tumours in nude mice.

2.3.2. Sunitinib (SU011248)

Sunitinib is administered orally and inhibits the receptor tyrosine kinases VEGFR2, PDGFR, FLT-3 and c-KIT with IC_{50} concentrations in the nanomolar range (Table 1) [56–58]. Two independent multicentre phase II trials of sunitinib in metastatic RCC have been reported. The first trial [59] recruited 63 patients and the second trial (ASCO 2005, Abstract 4508, Motzer et al.) recruited 106 patients; eligibility for both trials included failure of one previous cytokine therapy. Sunitinib was given as a single agent at 50 mg daily for 4 weeks followed by a 2 weeks rest period. Twenty-five patients (40%) in the first trial achieved a partial response (PR) and 17 (27%) had stable disease for at least 3 months. Median time to progression in the 63 patients was 8.7 months. Fatigue was the commonest adverse event; 27% of patients had grade 2 and 11% of patients had grade 3 fatigue. All other grade 3 clinical adverse events had an incidence of less than 5% whilst grade 2 diarrhoea, nausea, constipation, dyspepsia, stomatitis or vomiting was reported in approximately 10–20% of patients. Eighty-three patients in the second trial were assessable for response at the time of reporting. Twenty-five patients (29%) had at least a 30% decrease in tumour size including 1 complete response, 16 confirmed partial responses and 7 unconfirmed partial responses. The high response rates and generally manageable toxicity profile of sunitinib as second-line therapy reported in these trials have prompted a phase 3 trial of sunitinib versus interferon as first-line therapy for metastatic renal cell carcinoma that has just completed recruitment.

2.3.3. Axitinib (AG-013736)

Axitinib is an oral inhibitor of the receptor tyrosine kinases VEGFR1, VEGFR2, PDGFR and c-KIT (Table 1) given at a dose of 5 mg twice daily [60]. A phase 2 study of axitinib in metastatic renal cell carcinoma has been reported in abstract form (ASCO 2005, Abstract 4509, Rini et al.). Fifty-two patients entered the study; eligibility criteria included failure of one prior cytokine-based therapy. A partial response to treatment was reported in 24 patients (46%). With a median follow up of 1 year, only 1 patient with a PR had relapsed (after 232 days of therapy). Reported grades 1 or 2 toxicities were nausea (29%), fatigue (29%), diarrhoea (27%), hoarseness (19%), anorexia (17%) and weight loss (15%). Hypertension was reported in 17 patients (33%); 12% of patients had grades 3 or 4 hypertension and 6% of patients had aggravated hypertension.

2.3.4. Sorafenib (BAY 43-9006)

Sorafenib is a bi-aryl urea that was designed as an inhibitor of the non-receptor serine threonine kinases BRAF and

CRAF. The BRAF and CRAF kinases are members of the RAF/MEK/ERK intracellular signaling cascade that is a downstream effector of RAS. RAS is activated in response to receptor tyrosine kinase stimulation and therefore potentially links signaling through VEGFR, PDGFR and EGFR to the RAF/MEK/ERK pathway. Activation of the RAF/MEK/ERK signaling cascade leads to changes in metabolism, transcription and cytoskeletal arrangements within the cell [61]. This pathway is known to be implicated in tumour cell survival, proliferation and angiogenesis and is a logical therapeutic target in cancer [62] but it is unknown to what extent aberrations in this pathway are important in renal cell carcinoma although there are some data to suggest it may relevant [63,64]. In addition to inhibition of BRAF and CRAF, sorafenib also inhibits the BRAF mutant V600E which is present in over half of malignant melanomas [65]. This mutation is not however reported in renal cell carcinomas [66].

In preclinical studies, sorafenib demonstrated antitumour activity in breast, colon and non-small cell lung cancer xenograft animal models [67]. Tumour growth retardation was associated with inhibition of the RAF/MEK/ERK pathway in two of the three xenograft models whereas significant inhibition of neovascularization was shown in all models. It was concluded therefore that sorafenib is a dual action RAF kinase and VEGFR inhibitor that targets both tumour cell proliferation and tumour angiogenesis. Like sunitinib and axitinib, sorafenib inhibits the receptor tyrosine kinases VEGFR2, VEGFR3, FLT-3, c-KIT and PDGFR with IC_{50} concentrations in the nanomolar range (Table 1). Four phase 1 studies in which 163 patients were treated identified 400mg twice daily continuous dosing as the recommended phase 2 dose of sorafenib [68–71].

The results of a multicentre phase 2 randomized discontinuation trial (RDT) of sorafenib have been reported in abstract form (ASCO 2005, Abstract 4544, Ratain et al.). The RDT design allows the antitumour activity of a study agent to be distinguished from slowly growing metastatic disease [72,73]. All patients initially are treated with the study agent (stage 1) and in those patients with stable disease, there is a double-blind randomization between continued therapy and placebo (stage 2). Patients with objective responses at the end of stage 1 continue the study agent until disease progression whilst therapy is discontinued in patients with progressive disease at the end of stage 1.

The phase 2 RDT of sorafenib in metastatic renal cell carcinoma evaluated the effect of the drug on tumour growth in patients with stable disease after 12 weeks of treatment in stage 1. Two hundred and two patients with advanced RCC were treated with sorafenib at a dose of 400 mg orally twice daily in stage 1. Most patients were receiving sorafenib as second-line (56%) or third-line (34%) therapy. All patients were PS 0 or 1 and 56% had undergone nephrectomy. Forty percent of patients responded to treatment at 12 weeks and 30% had progressive disease. Sixty-five patients with stable disease (response between 25% tumour reduction and 25% tumour growth) at 12 weeks were randomized to sorafenib ($n=32$) or placebo ($n=33$); patient characteristics were well matched between the groups. After 24 weeks, 6 patients (18%) on placebo were progression-free compared with 16 patients (50%) on sorafenib ($p=0.0077$). Median progression-free survival (PFS) after randomization was greater with sorafenib than placebo (23 weeks versus 6 weeks, $p=0.0001$, hazard ratio 0.29). Sorafenib was restarted in the 25 patients who progressed on placebo after a median time from randomization of 7 weeks. Median progression-free survival after restarting sorafenib in these patients was 24 weeks and 13 patients remained on therapy at the time of reporting. The most common drug-related toxicities in all 202 patients were rash (62%), hand-foot skin reaction (61%) and fatigue (56%). The authors conclude that that sorafenib has a marked effect on progression-free survival in metastatic renal cell carcinoma and an acceptable toxicity profile and that the trial demonstrates the utility of the RDT design.

The results of a multicentre phase 3 randomized double-blind trial of sorafenib in advanced renal cell carcinoma have also been updated in abstract form (ASCO 2005, abstract 4510, Escudier et al.). The primary aim of the trial was to assess overall survival (OS) in patients with advanced clear cell RCC randomized to sorafenib versus best supportive care. The results of a planned analysis on the secondary endpoint, progression-free survival (PFS) were reported. Subjects were again of PS 0 or 1, had received one prior systemic therapy for advanced RCC and were randomized to receive continuous oral sorafenib 400 mg twice daily or placebo with best supportive care. At the time of the progression-free survival analysis, 769 of the planned 884 patients had been randomized and 342 progression-free survival events had been reported. Baseline prognostic characteristics were similar between both groups; 82% of patients had received prior cytokine therapy and 93% had had prior nephrectomy.

Median progression-free survival was 24 weeks for sorafenib versus 12 weeks for placebo (hazard ratio 0.44; $p<0.00001$) and the 12-week progression-free rate was 79% for sorafenib versus 50% for placebo (Fig. 3). Drug-related toxicities for sorafenib versus placebo were rash (34:13%), diarrhoea (33:10%), hand-foot skin reaction (27:5%), fatigue (26:23%) and hypertension (11:1%). Grades 3 or 4 adverse events were reported in 30% of patients on sorafenib versus 22% of patients on placebo. The authors conclude that sorafenib significantly prolongs progression-free survival compared with placebo in patients with previously treated advanced renal cell carcinoma with an acceptable toxicity profile.

Further data from this study were recently presented (ECCO 2005, Oral presentation, Escudier et al.). By the data cut off at the end of May 2005, 903 patients had entered the trial. There was 1 complete response (in the sorafenib arm) and 51 partial responses (43 in the sorafenib arm and 8 in the placebo arm, 10% versus 2%) to treatment. Progressive disease was reported in 56 patients in the sorafenib arm and 167 patients in the placebo arm (12% versus 37%) and stable disease in 333 patients in the sorafenib arm and 239

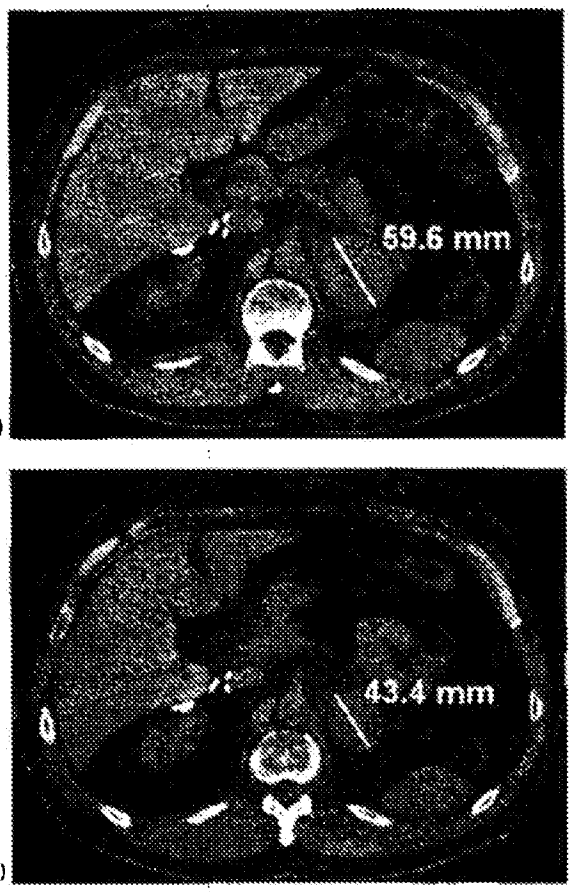

Fig. 3. Tumour shrinkage in response to sorafenib after 6 weeks of therapy (reproduced with permission Dr B. Escudier).

patients in the placebo arm (74% versus 53%). Interestingly, some of the patients with stable disease in the sorafenib had tumour shrinkage insufficient to meet the RECIST criteria for a partial response. Preliminary overall survival data from a planned interim analysis were also presented: median overall survival in the placebo group was 14.7 months and had not been reached in the sorafenib group (HR 0.72, $p = 0.018$). This was not a statistically significant difference as the threshold for significance for this analysis was $p < 0.0005$.

2.4. The mammalian target of rapamycin (mTOR)

2.4.1. mTOR in metastatic renal cell carcinoma

The mammalian target of rapamycin (mTOR) is a non-receptor tyrosine kinase; mTOR activation has various downstream effects including increasing HIF-1α gene expression at both the levels of mRNA translation and protein [74]. Inhibition of mTOR activation with contingent reduction in HIF-1α and pro-angiogenic cytokines is therefore a rational therapeutic strategy in renal cell carcinoma. Futhermore, reduced PTEN (phosphatase and tensin homologue deleted on chromosome 10) expression has been demonstrated in a proportion of renal cell carcinomas [75,76]. Loss of PTEN function results in Akt phosphorylation with downstream effects on cell survival, cell growth and proliferation that may be blocked using rapamycin derivatives [77] in this specific subset of tumours.

2.4.2. Temsirolimus (CCI-779)

Temsirolimus (CCI-779) is an mTOR inhibitor (Table 1) that has been evaluated in a phase 2 study in metastatic renal cell carcinoma in 111 patients who were randomly assigned to receive 25, 75 or 250 mg of the drug as a weekly intravenous infusion [78]. Over 90% of patients had had prior systemic therapy. One complete response, 7 partial responses (response rate = 7%) and 29 minor responses (26%) to treatment with temsirolimus were reported. Just over half of patients (51%) had a response to treatment or stable disease for over 24 weeks. The most frequent grades 3 or 4 toxicities were hyperglycaemia (17%), hypophosphataemia (13%), anaemia (9%) and hypertriglyceridaemia (6%). Neither toxicity nor efficacy was significantly influenced by temsirolimus dose level. Preliminary data have demonstrated that temsirolimus can be administered in combination with IFN-α without excessive toxicity and the results of a three-arm phase 3 study comparing temsirolimus, IFN-α and the combination of the two agents in metastatic renal cell carcinoma are awaited.

3. Future of kinase inhibitors in renal cell carcinoma

The kinase inhibitors sunitinib and sorafenib have demonstrated impressive activity as single agents in the second-line setting in advanced renal cell carcinoma and phase 3 trials comparing these drugs with interferon as first-line therapy are underway. If kinase inhibitors show superior efficacy to interferon as first-line therapy for metastatic disease, several important questions will remain.

A fundamental issue is that the true mechanism of action of kinase inhibitors has not been established in renal cell carcinoma in vivo. It is also unknown whether these drugs are active at all sites of disease or for example, cross the blood brain barrier, although there are good data to suggest that imatinib penetrates CSF poorly [79–84].

A further question is whether or not kinase inhibitors can be combined synergistically with immunotherapy and the mTOR inhibitor temsirolimus is under investigation in this setting. The data reported from a small study of the combination of the EGFR inhibitor erlotinib and the anti-VEGF antibody bevacizumab [17] appear not to have been confirmed in an unpublished randomized phase 2 study comparing bevacizumab with both drugs combined. A preliminary analysis reported in a press release on the Genentech website (www.gene.com) has shown similar response rates and progression-free survival for both arms of the study. This result raises questions about how kinase inhibitors, immunotherapy and antibody therapy can best be combined ('horizontally') in renal cell carcinoma.

The question of how systemic treatment might be sequenced ('vertically') is of equal importance. Controversy still surrounds the optimum immunotherapy of metastatic renal cell carcinoma [85] and there is a need for the identification of biomarkers that may allow the selection of patients for different therapeutic approaches. Carbonic anhydrase IX expression has emerged as a predictor of outcome in renal cell carcinoma patients receiving interleukin-2-based therapy [33] and there is interest in the use of microarray technology to classify renal cell carcinomas [86,87].

An interesting aspect of kinase inhibitor therapy with imatinib for GIST is that the drug dose can be increased safely on disease progression and that approximately a quarter of patients will experience renewed disease stabilisation as a result [88]: this begs the question of whether the same strategy can be adopted with kinase inhibitors in renal cell carcinoma; a related question is whether or not cross-resistance between different kinase inhibitors occurs in RCC. In addition to sorafenib, sunitinib and axitinib, a number of other VEGFR/PDGFR inhibitors such as PTK787, GW786034 and XL999 are under evaluation in metastatic RCC and most major pharmaceutical companies have at least one such drug in development. Although it is possible that kinase inhibitors in early development will have greater efficacy in metastatic RCC than the current generation of agents, it is perhaps more likely that activity in resistant settings and reduced toxicity will drive drug development. Mutational status in c-KIT and PDGFRA in GIST predict response to treatment with imatinib [89] and it is conceivable that the mutational status of VHL for example, may be used in the future as a marker of resistance or sensitivity to a given agent in metastatic RCC.

A further area for the investigation of kinase inhibitors in renal cell carcinoma is as adjuvant therapy. Although immunotherapy has proven efficacy in metastatic disease, no benefit has so far been demonstrated in the adjuvant setting [90–92] and in fact inferior overall survival has been reported recently with immunotherapy in comparison with placebo [93]. Given the activity of kinase inhibitors in advanced disease, there is a logical interest in evaluating these drugs in early stage disease to minimize the likelihood of relapse after surgery. An area of particular interest is the use of neo-adjuvant kinase inhibitors. Such an approach may allow the elucidation of the in vivo mechanism of action of kinase inhibitors in renal cell carcinoma; tumour downstaging may also occur, biomarkers of response prediction may be identified and radiological appearances correlated with pathological changes (e.g. what are the molecular correlates of central necrosis as seen on computed tomography? (Fig. 4)) [94]. There is, as a result, considerable enthusiasm for the investigation of kinase inhibitors in patients with metastatic renal cell carcinoma in the pre-operative setting [95].

The most important question for patients taking kinase inhibitors is the impact of the drugs on quality of life. In comparison with cytotoxic chemotherapy and in common with endocrine therapies, kinase inhibitors are often cytostatic; disease stabilisation therefore is an important efficacy endpoint. The corollary of this is that patients may often take kinase inhibitors for prolonged periods of time (compare hormones and cytotoxics) and toxicity is therefore a key issue. For example, sunitinib often causes significant fatigue whilst skin toxicity is probably the most problematic side effect of sorafenib and toxicity profiles may guide therapeutic decisions alongside molecular, pathological and clinical factors.

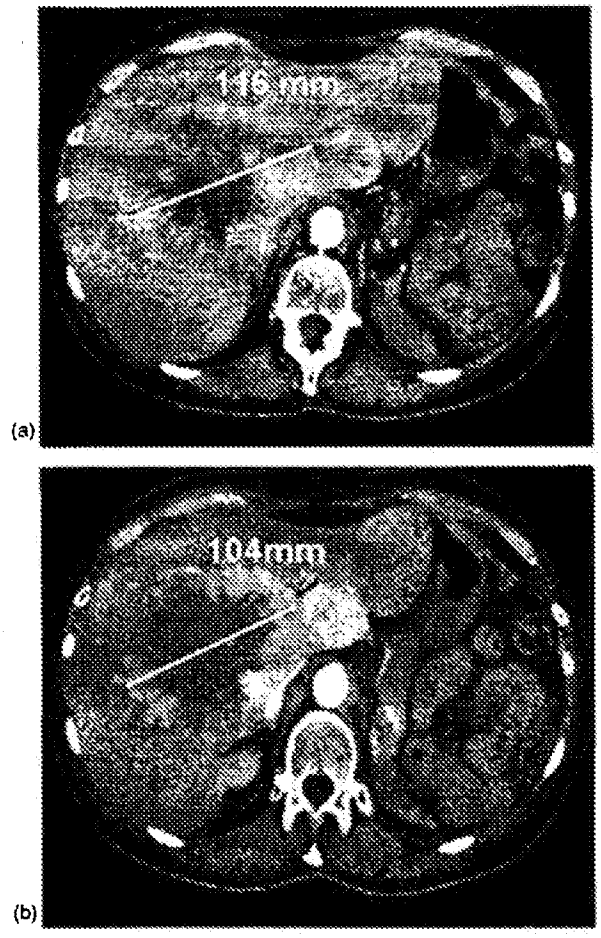

Fig. 4. Central necrosis of a metastatic lesion after sorafenib therapy (reproduced with permission Dr B. Escudier).

4. Conclusions

Nephrectomy can be curative in localized renal cell carcinoma and has a role in the management of advanced disease. Immunotherapy with interleukin-2 or interferon-α results in a small overall survival advantage in metastatic disease but a need exists for better systemic therapies. Pathological angiogenesis has a key role in renal clear cell carcinoma and kinase inhibitors that interfere with this process have demonstrated efficacy in phase 2 studies in advanced disease.

The small molecules sunitinib, sorafenib and axitinib inhibit the vascular endothelial growth factor (VEGF),

platelet-derived growth factor (PDGF) and c-KIT receptor tyrosine kinases whilst temsirolimus inhibits the mammalian target of rapamycin (mTOR). These drugs usually are orally administered and have shown significant activity with manageable toxicity in phase 2 studies in metastatic RCC in generally immunotherapy-refractory patients. Sorafenib has demonstrated prolonged progression-free survival in a phase 3 study in comparison with placebo. Overall survival data from this trial are awaited and other phase 3 trials comparing sunitinib, sorafenib and temsirolimus with interferon as first-line therapy are underway.

Given the activity of kinase inhibitors in advanced renal cell carcinoma, these agents are likely to be investigated in the adjuvant setting to attempt to minimize the risk of disease recurrence and in the neoadjuvant setting to identify biomarkers of response to therapy, to investigate mechanisms of drug action *in vivo* and potentially to downstage tumours prior to surgery.

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Biographies

James Larkin trained in medicine at Cambridge University and at the University of Oxford School of Clinical Medicine, graduating in 1996. He completed 3 years of general medical training and became a member of the Royal College of Physicians in 1999. Between 2001 and 2004 he was a Medical Research Council clinical fellow at the Institute of Cancer Research in London. In 2005 he was awarded a Ph.D. for laboratory work on the preclinical modelling of anti-vascular therapy for cancer. Currently he is training as a medical oncologist at the Royal Marsden Hospital.

Tim Eisen is trained in medicine at the Middlesex Hospital Medical School, London and University of Cambridge School of Medicine and graduated MB BChir in 1986. He completed 4 years of general medical training and became a member of the Royal College of Physicians (UK) in 1990. Between 1991 and 1995 he was a clinical research fellow at the Marie Curie Research Institute in Surrey. During this time he completed a Ph.D. on the molecular biology of melanoma. Following this he trained as a medical oncologist on the Senior Registrar rotation of the Royal Marsden Hospital, London and Surrey. He received his Certificate of Completion of Specialist Training in Medical Oncology in 1997. He became a fellow of the Royal College of Physicians in 2002. In March 1998 he was appointed as Senior Lecturer in Medical Oncology at University College London where he developed special interests in the treatment of kidney cancer, melanoma and lung cancer. In February 2001 he took up a new post as Senior Lecturer in Medical Oncology at the Institute of Cancer Research and the Royal Marsden Hospital. His clinical responsibilities include the management of kidney cancer, melanoma and lung cancer. He was appointed chairman of the NCRI Lung Clinical Studies Group in 2004. He was appointed as Professor of Medical Oncology in Cambridge in December 2005 and aim to take up his post in 2006.

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Abstract

A phase I study of axitinib (AG-013736), a potent inhibitor of VEGFRs, in combination with gemcitabine (GEM) in patients (pts) with advanced pancreatic cancer

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13092

Background: Axitinib (AG-013736) is a novel small molecule inhibitor of the receptor tyrosine kinases with picomolar potency against VEGFR 1, 2 & 3 and nanomolar potency against PDGFR-beta and KIT. A phase I study in solid tumors identified 5 mg BID as the therapeutic dose; a phase II study in renal cell cancer demonstrated significant efficacy with a response rate (RR) of 46% (Rini et al, ASCO 2005). This study examined the safety, PK and preliminary efficacy of AG-013736 (AG) in combination with gemcitabine (GEM) as first-line therapy for advanced pancreatic cancer. **Methods:** A randomized phase II study was preceded by a phase I component. All patients (pts) in the phase I portion received 1000 mg/m^2 GEM by 30-minute infusion on days 1, 8, and 15 followed by one week of rest from treatment. AG 5 mg p.o. BID was given beginning Cycle 1, Day 3 (C1D3). Eligible pts had no prior chemotherapy for advanced disease, ECOG 0–2, and no previous treatment with VEGF/VEGFR inhibitors, or anti-angiogenesis treatment. Full PK profiles were collected on C1D1 (GEM alone), C1D14 (steady state, AG alone), and C1D15 (GEM + AG). In the phase II trial, pts are randomized to AG or AG plus GEM beginning C1D1. **Results:** 8 pts were treated on the phase I portion of this trial. Toxicity: The primary Gr. 3/4 toxicity was hematologic: Gr. 4 anemia and Gr.3 thrombocytopenia in 1 pt and Gr. 3 neutropenia in 1 pt requiring a dose reduction for GEM in Cycle 3. Gr. 2 non-hematologic adverse events include pruritus (1 pt), abdominal pain (2 pts), epigastric pain (1 pt), melena (1 pt), and asthenia (2 pts). Gr. 2 hypertension was observed in 3 pts. Efficacy: Radiological assessment suggests 2 pts with partial response and 4 pts with stable disease: response assessments are ongoing. The median number of cycles is 3 [1,6]. Treatment for 4 pts is still ongoing: Cycle 6 (2 pts) and Cycle 2 (2 pts).

Conclusions: This combination is safe and appears to be an effective treatment for advanced pancreatic cancer with significant tumor regression observed in 2 pts. Therapy was well tolerated with manageable toxicity. Additional investigation of AG-013736 in combination with GEM in the phase II setting for advanced pancreatic cancer is warranted.

Author Disclosure

Employment or Leadership	Consultant or Advisory Role	Stock Ownership	Honoraria	Research Funding	Expert Testimony	Other Remuneration
Pfizer						

A Phase 2 study of Axitinib (AG-013736), a potent inhibitor of VEGFRs, in patients with advanced thyroid cancer

Abstract 5529

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Abstract

Background: Elevated levels of VEGF have been found in thyroid tumor tissue compared to normal thyroid. Axitinib (AG-013736) is a novel small molecule inhibitor of the receptor tyrosine kinases with picomolar potency against VEGFR 1, 2 & 3 and nanomolar potency against PDGFR-ß and KIT. A phase I study in solid tumors identified 5 mg BID as the therapeutic dose. A phase II study in renal cell cancer demonstrated significant efficacy with a response rate (RR) of 46% (Rini et al, ASCO 2005). This study examined the safety and efficacy of AG-013736 (AG) therapy for advanced thyroid cancer. Methods: 32 patients (pts) refractory to or not suitable candidates for Iodine (^{131}I) treatment were treated with AG 5 mg p.o. BID. Eligibility included measurable disease and ECOG performance status (PS) of 0 or 1. A Simon 2 stage minimax design was used (alpha=0.1; beta=0.1; null response rate (RR)=5%; alternative RR=20%). Results: The median age was 62 yrs (30-84). 19 (59%) were male. 13 (41%) were female. Histological subtypes include papillary: 17 pts (53%); follicular- 9 pts (28%)- 8 with Hurthle cell variant, medullary- 3 pts (9%), anaplastic-2 pts (6%) and other- 1pt (3%). Twenty-nine pts (91%) had prior surgery, 19 pts (59%) had prior ^{131}I treatment, 15 pts (47%) had prior RT, 6 pts (19%) had prior chemotherapy. Best response by RECIST per investigator report indicate PR in 7 pts (22%) with a maximum tumor regression ranging from 36-67% and additional unconfirmed PR in 2 pts (6%). Two pts (follicular) have PR >12 months. An independent radiology review confirms PR in 3 pts (9%) and an additional unconfirmed PR in 3 pts (9%). Response assessments are ongoing. 14 pts have discontinued treatment: 6 pts (19%)-progression, 5 pts-adverse events (AEs) and 3 pts-other. The range for time on study is (6, 517) days. Median progression free survival has not been reached. The most common treatment related adverse events are fatigue (47%), diarrhea (31%), nausea (31%), proteinuria (38%), hypertension (28%), anorexia (25%), decreased weight (19%), stomatitis (19%), mucosal inflammation (19%) and vomiting (19%). Conclusions: AG-013736 has substantial anti-tumor activity in advanced thyroid cancer. Therapy is well tolerated with manageable toxicity. Further investigation in this setting is warranted.

updated

Target Affinities of Axitinib

Target	Cell Potency (IC_{50}, nM)
VEGFR-2	$IC_{50} = 0.16$
VEGFR-1	$IC_{50} = 1.2^{*}$
VEGFR-3	$IC_{50} = 0.29$
PDGFR-β	$IC_{50} = 1.6$
KIT	$IC_{50} = 1.7$
PDGFR-α	$IC_{50} = 5.2$
CSF-1R	$IC_{50} = 49$
FGFR-1	$IC_{50} = 218$
Flt3	$IC_{50} > 1000$

*Low serum assay conditions employed for all assays except VEGFR-1.

Objectives

Primary

- Determine the activity of AG-013736 in advanced thyroid cancer as measured by the overall response rate (complete and partial response rate by RECIST)

Secondary

- Safety profile of AG-013736
- Progression-free survival (PFS)
- Duration of response
- Overall survival (OS)
- Population pharmacokinetic analyses

Phase II Trial

Design

- Single-arm, multi-center, IRB-approved trial
- Primary endpoint of overall response rate ($H_o = 5\%$, $H_a = 20\%$)
- 2-stage design, target accrual of 32 patients
- Additional cohort of 28 patients to be enrolled if 4/32 responses observed

Treatment

- AG-013736 (starting dose): 5mg orally twice daily continuously until disease progression or unacceptable toxicity
- Radiographic assessment at baseline and Q 8 weeks
- Response assessed by RECIST

Major Eligibility Criteria

- Histologically documented thyroid cancer with metastases
- Failure of ^{131}I to control the disease or lack of iodine uptake
- No expectation of further effects of prior anticancer therapy
- At least 1 target lesion, as defined by RECIST that has not been externally irradiated
- Adequate bone marrow, hepatic, and renal function
- Age ≥18 years
- ECOG performance status of 0 or 1
- No evidence of preexisting uncontrolled hypertension

Patient Demographics (N=32)

Characteristics	*No.*	*%*
Median Age (Range)	62 (30-84)	
Male/Female	19/13	*59/41*
ECOG PS (0/1)	10/22	*31/69*
Histology		
Papillary	17	*53*
Follicular/ Hurthle cell variant	9/8	*28/25*
Medullary	3	*9*
Anaplastic	2	*6*
Other	1	*3*
Prior Surgery	29	*91*
Prior ^{131}I/ Radiation Therapy	19/15	*59/47*
Prior Chemotherapy	6	*19*

Patient Demographics (N=32)

Characteristics	*No.*	*%*
Sites of Disease		
Lung	27	84
Lymph Node	16	50
Thyroid Bed	15	47
Bone	11	34
Liver	6	19

Results

Common Treatment-Related AEs > 10%

Adverse Event	*Total, N (%)*	*Grade 3 N (%)**
Fatigue	15 (47)	3 (9)
Proteinuria	12 (38)	1 (3)
Diarrhea	10 (31)	1 (3)
Nausea	10 (31)	1 (3)
Hypertension	9 (28)	2 (6)
Anorexia	8 (25)	0
Weight Decreased	6 (19)	1 (3)
Stomatitis	6 (19)	0
Mucosal Inflammation	6 (19)	0
Vomiting	6 (19)	0

*No Grade 4 treatment related adverse events reported

Dose reductions in 9 (28%) patients due to adverse events

Patient Status (N=32)

	No.	%
On Treatment	18	56
Treatment Discontinuation	14	43
Disease Progression	6	19
Adverse Event (Disease Related)*	3	9
Adverse Event (Treatment Related) *Proteinuria and Subject withdrawal due to hypertension*	2	6
Other**	3	9

Median Treatment Duration (days): 185 [6, 517]

*includes 2 deaths due to disease under study

**includes 1 death due to unknown cause

Best Response by RECIST (N=32)
Investigator Report

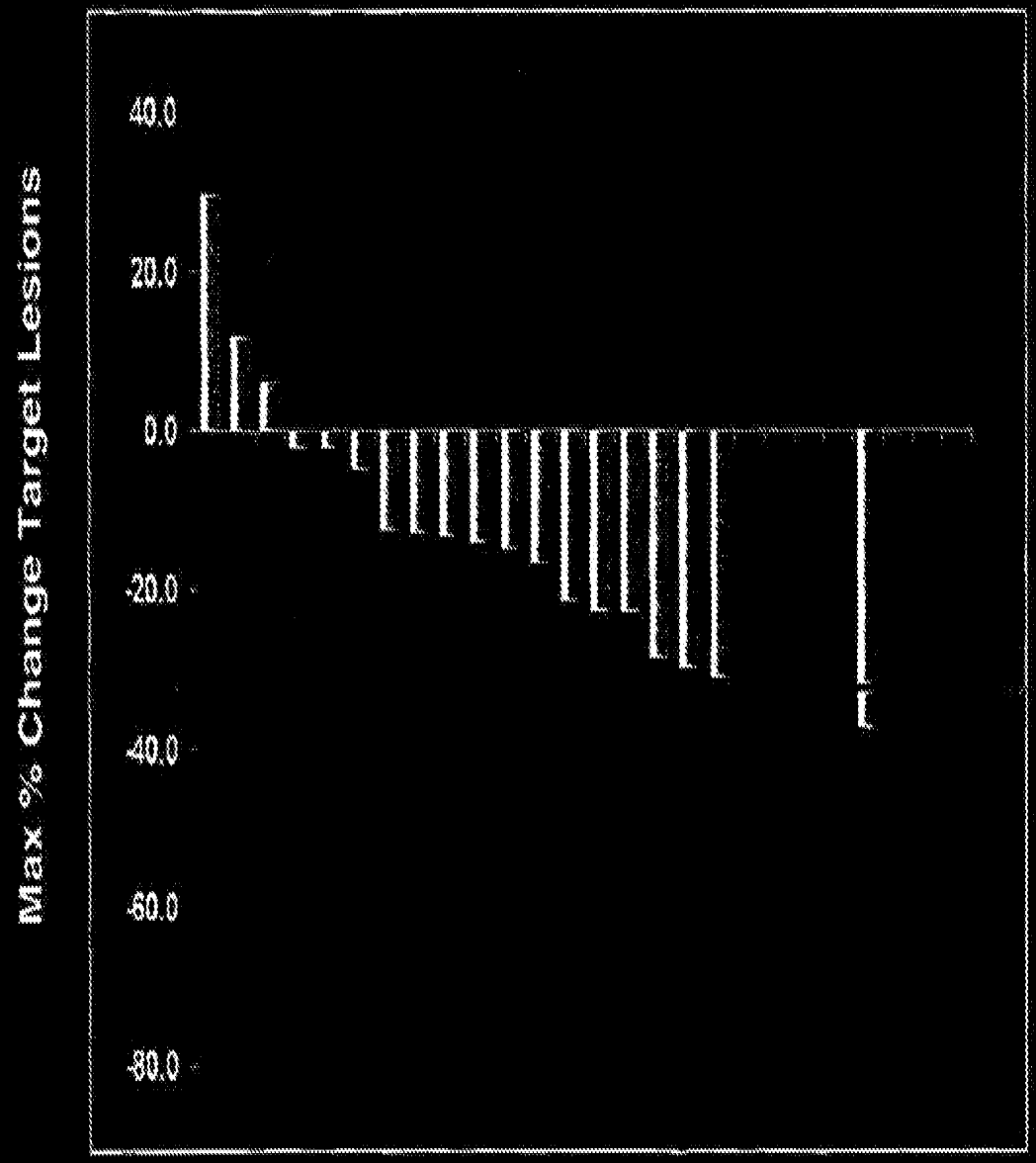
	No.	%
Partial Response	7	22%
	95% CI (9.3%, 40%)	
*Stable Disease**	15	47%
No. with any Tumor Shrinkage	14**	93
No Response	10	%
Progression	5	15
Indeterminate	5	15

*SD defined as ≥ 16 weeks
**includes 2 unconfirmed PR

Best Response by Histology

Histology (n)	*PR (N=7)*	*SD/PD (N=20)*
Follicular (9)	4	5/0
Papillary (17)	2	9/3
Anaplastic (2)	1	0/1
Medullary (3)	0	0/1
Other (1)	0	1/0

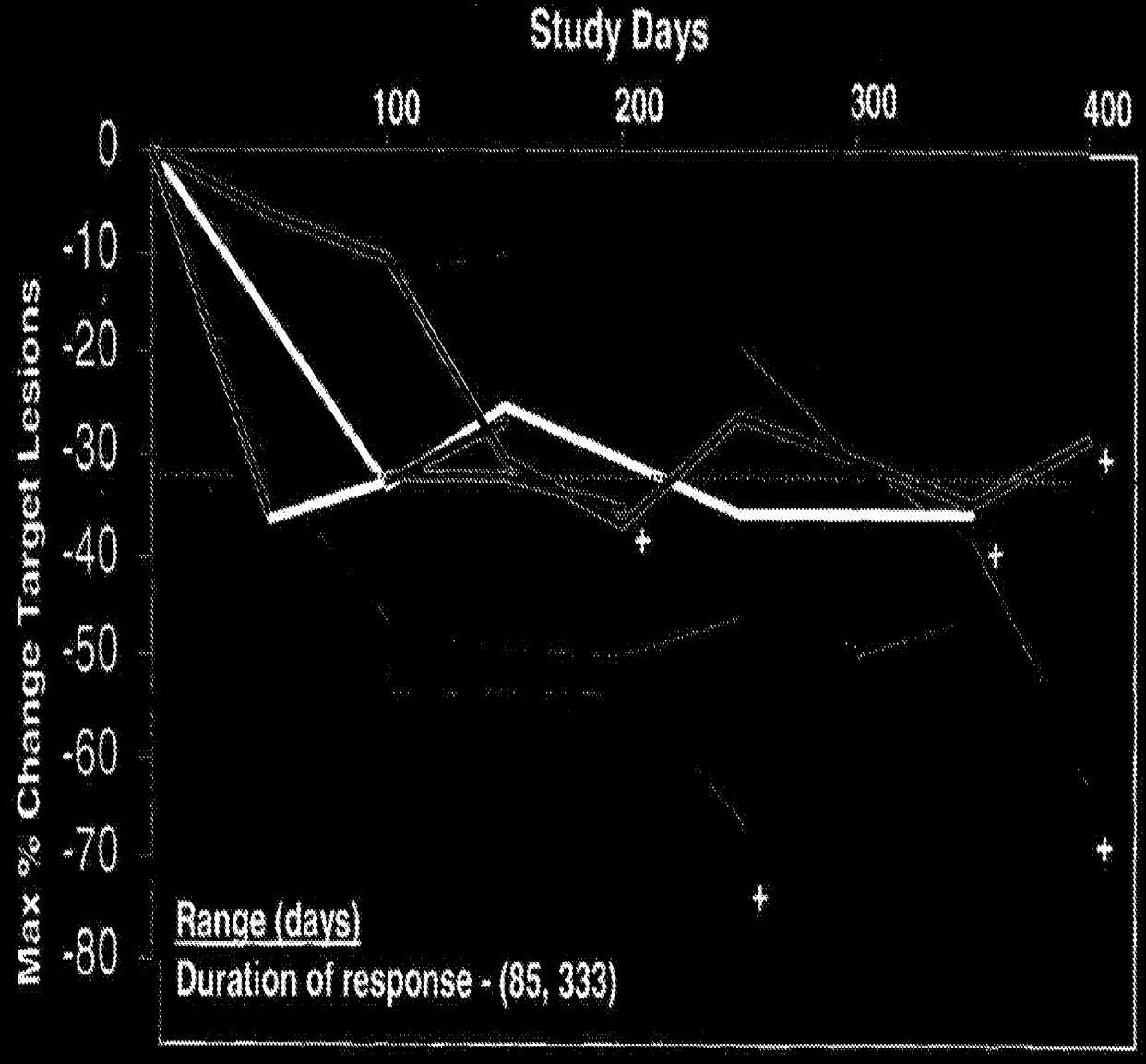
Maximum % Reduction in Target Lesions (N=32) by Investigator Report



*Excludes 5 patients without a post-baseline scan and 1 patient-indeterminate
3 PR have been confirmed by independent review

Target Lesion Shrinkage Over Time

PR Patients Only, (N=7)



+ Patients ongoing (5)

Conclusions

- Axitinib, a potent oral inhibitor of all VEGF receptors, demonstrates anti-tumor activity in ^{131}I refractory thyroid cancer as evidenced by a substantial objective response/ tumor shrinkage rate and duration of response range of 85 - 333 days.
- 7 out of 32 (22%) have achieved partial response (PR) by investigator report.
- 15 subjects (47%) have sustained disease stability (SD) with a range of 4 - 13 months.
- Axitinib is well tolerated with manageable toxicities. Most common treatment related adverse events are fatigue, proteinuria diarrhea, nausea, and hypertension.
- Further investigation of Axitinib in advanced thyroid cancer is warranted.